

Characterising pesticide residues in floral resources for bees

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and is entirely my own work except where duly indicated and clearly acknowledged in the text.

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A handwritten signature in blue ink, consisting of a stylized 'E' and 'Z' followed by a small dot.

Elena Zioga

"The aim of science is not to open the door to infinite wisdom, but to set a limit to infinite error."

Bertolt Brecht

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"We shall not cease from exploration and the end of all our exploring will be to arrive where we started and know the place for the first time."

Thomas Stearns Eliot

Summary

Pesticide use in agriculture is one of the main factors driving pollinator declines. Bees are highly important pollinators and may be at risk from exposure to pesticide compounds while foraging for pollen and nectar. In this thesis, I explored the extent of pollen and nectar contamination by pesticides used in agriculture.

Worldwide knowledge gaps were identified via a novel systematic literature review, which summarised and critically evaluated pesticide residue levels and occurrence in nectar and pollen directly collected from plants. Through this review, it became clear that most previous work had focused on detecting neonicotinoid insecticide residues, more often examining pollen rather than nectar, and that residues of several compounds were estimated to exceed the lethal dose for several bee species. However, studies were geographically constrained, and the range of compounds evaluated so far was narrow.

Given this insufficient understanding of the extent to which different compounds may be detected in pollen and nectar, I compared the mixtures and levels of pesticide residues in pollen and nectar of crops and wild plants growing in the margins of the crops in Ireland, whose land use is dominated by agriculture. Based on their application (volume and frequency) and relevance to bee health, the fungicides azoxystrobin, boscalid and prothioconazole, the herbicides fluroxypyr and glyphosate (and its main metabolite AMPA), and five neonicotinoid insecticides (acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam), were identified as the targeted analytes for this residue evaluation study. As model crop species I used oilseed rape (*Brassica napus* L.), while blackberry plants (*Rubus fruticosus* agg.) were used as an example wild plant species.

Overall, more residues were detected in *B. napus* pollen and nectar than in the wild plant, and *B. napus* pollen had the highest mean concentration of residues. My results demonstrated that most detections were from fields with no recorded application of the respective compounds in that year, but higher concentrations were observed in recently treated fields. The most frequently detected compounds were fungicides, and all matrices were contaminated with at least three compounds. The most common compound mixtures were two fungicides and one neonicotinoid insecticide not recently applied on the cultivated fields. This indicated that persistent compounds like the neonicotinoids, should be continuously monitored for their presence and fate in the field environment.

Following this, I compared the mixtures and levels of pesticide residues in pollen of different crops and corbicular pollen from honey bees and bumble bees foraging simultaneously under the same agricultural conditions. For this, as model crop species I used oilseed rape (*Brassica napus* L.) and broad bean (*Vicia faba* L.), while pollen foragers of the common honey bee (*Apis mellifera* L.) and various wild bumble bee species (e.g., *Bombus lucorum* agg., *B. lapidarius* etc.) were collected from crops.

Samples from *B. napus* fields had higher number of pesticide detections, higher residue concentrations and higher number of different pesticide compounds detected compared to those from *V. faba* fields and the compounds azoxystrobin, boscalid and thiamethoxam formed the most common pesticide mixture. Crop pollen was contaminated only with fungicides, honey bees collected pollen mainly with fungicides, while bumble bee pollen had more insecticide detections. The highest number of compounds and most detections were observed in bumble bee collected pollen, where notably, all the five neonicotinoids assessed (acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam) were detected despite the European ban and no recent application on the studied fields. The concentrations of neonicotinoid insecticides were positively correlated with the number of wild plant species present in the bumble bee pollen samples. These results raise concerns about potential bee exposure to multiple residues and whether honey bees are suitable surrogates for pesticide risk assessments for all bee species.

Finally, since glyphosate and its main metabolite AMPA have different physicochemical properties compared to the rest of the evaluated compounds, I evaluated them using separate analytical methods specifically designed for polar pesticides that I optimised. Their occurrence and levels in crop and wild plant pollen and nectar, as well as honey bee and bumble bee collected pollen under realistic agricultural conditions were evaluated for the first time. My results indicate off-target contamination of non-target plant pollen and nectar raising concerns for possible implications of glyphosate residues detected in nectar, connected to bee and human health.

This work establishes a baseline of knowledge on the pesticide contamination levels of matrices relevant to bee species, such as pollen and nectar directly collected from plants, and honey bee and bumble bee corbicular pollen. Future research can draw upon the findings of this research and expand our understanding of pesticide residue fate in an agricultural context and assess whether bees and other beneficial insects are affected by pesticide exposure, and to which

extent. This consequently can apprise ecologically friendly agricultural practices and inform policies, which will contribute to a sustainable pesticide use for future generations.

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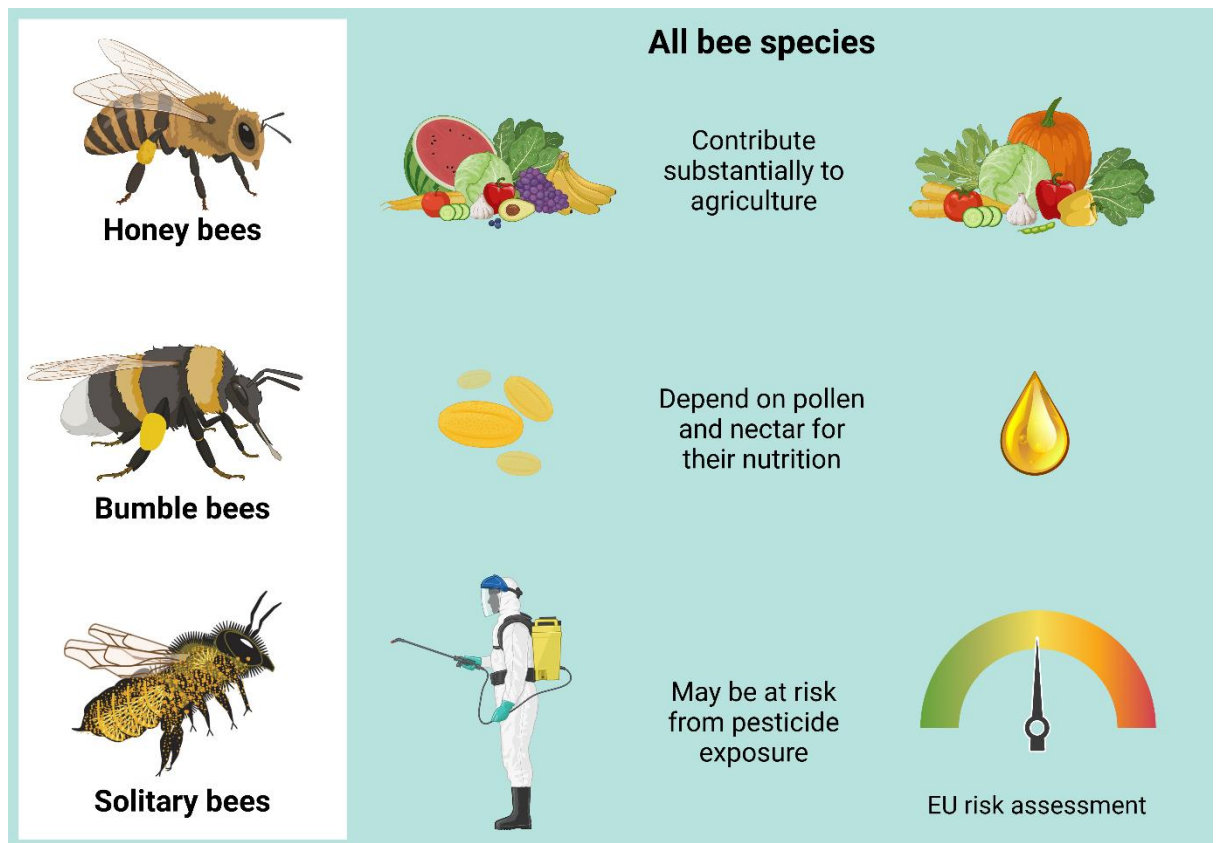
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Chapter 1

General introduction



1. General introduction

1.1. Bee importance and decline

Pollination occurs when pollen is transferred from the male flower part, the anther, to the female part, the stigma. In some cases, the flower's appearance, scent, and nectar production are often adapted to attract the particular pollinators that are most effective for that species (Kessler et al., 2015). Most European flowering plant species are animal pollinated (Ollerton et al., 2011), and of the 100 crops that provide 90% of the world's food supply, 71 are bee pollinated (Klein et al., 2007). Specifically in Europe, 84% of the 264 crop species are animal pollinated and 4,000 vegetable varieties exist thanks to pollination by bees (UNEP, 2010; Williams, 1994). Across Europe, crop pollination accounts for nearly €15 billion of the total economic value of crop production per year (Leonhardt et al., 2013). In Ireland, Stanley et al. (2013) estimated the overall value of pollination services to oil seed rape alone was €3.9 million per year and the annual value of animal pollination to home produced food crops was recently estimated to be up to €59 million per year (Stout et al., 2019).

Wild pollinators, through the pollination services they provide, are important globally for healthy human diets, agricultural economies, and nutritional security (IPBES, 2016). Insect pollinators, through the plants they pollinate, play important ecological roles beyond cropping systems by providing fruits and seeds for birds and other wildlife, contributing to nutrient cycling (e.g., nitrogen fixation), and to climate change adaptation, while many other beneficial taxa are favored under pollinator conservation measures (All Ireland Pollinator Plan, 2021). Worldwide research has shown that crop pollination and yields increase under more diverse and abundant pollinator presence in the agricultural environment (Dainese et al., 2019; Woodcock et al., 2019). Pollinator diversity ensures that plants will be pollinated even in cases where certain species fail to perform. It enables resilience to the ever-changing environment and acts as a buffer in case of unforeseen or uncertain major changes, especially in the context of climate change (European Commission, 2023b). Thus, it is essential to conserve pollinator species diversity and maintain their population numbers.

Globally most pollinators are wild species, and although non-bee pollinators (flies, beetles, moths, butterflies, birds, and bats, among others) also play an important role in pollination (Rader et al., 2016), bees are the most important pollinators because they are dominant pollinators in many habitats across the world (Klein et al., 2003; Stanley et al., 2013; Willmer, 2011; Winfree et al., 2008) and their entire life cycle is dependent on interactions with flowering

plants, thus, contributing substantially to the act of pollination (All-Ireland Pollinator Plan, 2021).

Widespread wild bee declines have been reported over the past several years (Powney et al., 2019; Sánchez-Bayo and Wyckhuys, 2019; Soroye et al., 2020; Zattara and Aizen, 2021), both in Europe (Biesmeijer et al. 2006, Carvalheiro et al. 2013; Kosior et al., 2007; Goulson et al., 2008; Vanbergen and Initiative, 2013) and North America (Burkle et al., 2013; Cameron et al., 2013; Mathiasson and Rehan, 2019). In Ireland, research has shown that more than half of the island's bee species have undergone substantial declines in numbers since 1980, with 30% of species considered threatened with extinction from Ireland according to IUCN criteria (Fitzpatrick et al., 2006). Three bee species considered threatened in Ireland are also threatened with extinction at the European level, and an additional four species are near threatened (Nieto et al., 2014). Several concurrent and interacting factors have been associated with bee declines (Goulson et al., 2015; Cameron and Sadd, 2020), including land use intensification and habitat loss (Goulson et al., 2015; Scheper et al., 2015), climate change (Flores et al., 2019; Memmott et al., 2007), infection by pathogens (Core et al., 2012; de Miranda and Genersch, 2010; Guzmán-Novoa et al., 2010; Higes et al., 2010; Morawetz et al., 2019; Smith et al., 2014), and exposure to pesticides (Belzunces et al., 2014; Bortolotti and Montanari, 2003; Desneux et al., 2007; Favaro et al., 2019; Goulson et al., 2015; Henry et al., 2012; Potts et al., 2010a,b, 2016; Yang et al, 2008).

1.2. Risk posed to bees by pesticides used in agriculture

A pesticide is any substance of chemical or biological nature used to prevent, control, or kill pests (Silva, 2022). These substances are very diverse being often classified based on their function, chemical composition, mode of entry, mode of action, hazard, formulation, or source of origin (Akashe et al., 2018). Most pesticide sales are linked to the agriculture sector (90%) (Antier et al., 2020), and are applied crops to prevent, or combat, plants that compete for resources with the crops, and/or to crops to combat organisms that damage the crops and agricultural commodities (Jess et al 2018; Krupke et al., 2012; Kudsk et al., 2018; Silva, 2022). Pesticides are beneficial to production, in that they provide crop protection, food preservation and disease control; however, they are toxic by design and some groups have been determined to pose serious risks to human health and the environment (WHO, 2022). Besides the farm productivity boost, pesticide benefits have been recognised for their contribution to food security and worldwide economy (Cooper and Dobson, 2007; Damalas, 2009; Nishimoto, 2019). Securing high yields is more important than ever given the projected increases in the human population (United Nations, 2019), the increasingly caloric diets, the rise in degradation and

depletion of arable lands, and the predicted impact of climate change on agriculture (Ray et al., 2019; Lykogianni et al., 2021).

However, when applied, pesticides are released into the environment (Carvalho, 2017) and, several are toxic to non-target species (Colin et al., 2019; Sánchez-Bayo, 2011; Ullah et al., 2018), persistent in the environment (Silva et al., 2019), and/or accumulate through food chains (Goutner et al., 2012; Wang et al., 2011). Thus, increasing pesticide sales intensified concerns about their sustainable use and raised the awareness of their negative impacts. First warnings on pesticide side-effects in the environment were raised more than half a century ago, through the landmark book, *Silent Spring* (Carson, 1962). A key priority for achieving sustainable global food production is to find a balance between protecting crops efficiently against pests and diseases and maintaining healthy pollinator populations (Godfray and Garnett, 2014). The current widespread use of pesticides may jeopardize the ecosystem services provided by insect pollinators by exposing them to mixtures of harmful chemicals (Botías et al., 2017; Godfray et al., 2014, 2015; Johnson, 2015; Krupke et al., 2012; Uhl and Brühl, 2019), especially in an agricultural context (Hallmann et al., 2017). Recent studies, based on field, semi-field, and laboratory experiments, on the impact of pesticides on wild bees (Baron et al., 2017a; Klaus et al., 2021; Rundlöf et al., 2015; Woodcock et al., 2017) have reported negative effects of pesticides on wild bee reproduction and consequently on their population growth (Powney et al., 2019; Willis Chan and Raine, 2021). Today numerous studies address the environmental and health risks of pesticides (Ali et al., 2021; Buckwell et al., 2020; Geiger et al., 2010; IPBES, 2016; Maggi et al., 2021), the hidden and external costs of pesticides (e.g., regulatory costs and defensive expenditures) (Bourguet and Guillemaud, 2016), and the pesticide agenda is getting more political, public and media attention (e.g., the case of neonicotinoid insecticides and bee poisoning).

1.2.1. Neonicotinoids and other insecticides

Neonicotinoids are the most widely used group of systemic insecticides first sold on the market in 1991, introduced as a safer alternative to older, more dangerous chemicals such as the organophosphates (Jeschke et al., 2010). They provide effective pest control in crop management as they bind strongly to the nicotinic acetylcholine receptors in the central nervous system of insects, altering synaptic functioning at low concentrations (Moffat et al., 2016) and causing receptor blockage, paralysis, and death at higher concentrations (Goulson, 2013; Matsuda et al., 2020). Over the past two decades, these widely used neurotoxic insecticides, have been the most studied pesticides in terms of potential negative impacts on bee health

(Blacquière et al., 2012; Crall and Raine, 2013; Goulson, 2013; Godfray et al., 2014, 2015; Lundin et al., 2015; Wood and Goulson, 2017). In addition to being highly toxic to honey bees and wild bees (Lewis et al., 2016), exposure to neonicotinoids can also have various sublethal effects on bees. Due to their wide application, persistence in soil, high water solubility and potential for uptake by succeeding crops and wild plants, pollinators are exposed to neonicotinoids in sublethal concentrations for many years after their last application on the fields (Bonmatin et al., 2015; Li, 2020; Long and Krupke, 2016; van der Sluijs et al., 2013; Wintermantel et al., 2020). In bees, neonicotinoid insecticides have been found to impair bee colony development and reproduction (Ellis et al., 2017; Feltham et al., 2014; Gill et al., 2012; Rundlöf et al., 2015; Sandrock et al., 2014; Whitehorn et al., 2012; Willis Chan and Raine, 2021; Woodcock et al., 2017), learning and memory functions (Stanley et al., 2015; Wright et al., 2015; Samuelson et al., 2016; Phelps et al., 2018; Muth et al., 2019), foraging (Yang et al., 2008; Gill et al., 2012; Feltham et al., 2014; Gill and Raine, 2014; Karahan et al., 2015; Stanley and Raine, 2016; Muth and Leonard, 2019; Willis Chan and Raine, 2021), flight (Fischer et al., 2014; Stanley et al., 2016; Tosi and Nieh, 2017), and homing behaviours (Henry et al., 2012). The impact of sublethal doses of neonicotinoid on offspring production of wild solitary bees is high, ranging from a reduction of nearly 40-100% (Rundlöf et al., 2015; Sandrock et al., 2013; Stuligross and Williams, 2020; Willis Chan and Raine, 2021). Moreover, clothianidin and thiamethoxam have adverse effects on the sperm viability of solitary mason bees (Strobl et al., 2021a, b) and thiamethoxam was found to impair bumble bee crop pollinations services (Stanley et al., 2015a). Because of the mounting evidence of negative effects of neonicotinoids on bees and other wildlife, and their long-term persistence in the environment, the use of some neonicotinoid compounds has been restricted in some parts of the world (Sgolastra et al., 2020). In Europe specifically, a complete ban on the agricultural use of the commonly used neonicotinoids clothianidin, imidacloprid, and thiamethoxam has been in place since 2018 and for thiacloprid since 2020 (European Commission, 2022a). Moreover, the European Court of Justice (ECJ) confirmed that European Member States will no longer be allowed to grant derogations temporarily permitting the use of seeds treated with neonicotinoids (ECJ, 2023).

Insecticides pose the highest risk for bee populations as they are designed to kill insects (Johnson, 2015; Sánchez-Bayo and Goka, 2014), and neonicotinoids are not the only insecticide class to have demonstrated negative effects on bees. Pyrethroids, a class of non-systemic insecticides, are also toxic to wild bees (Baron et al., 2014; Ceuppens et al., 2015; EFSA et al., 2018; Spurgeon et al., 2016) and can induce sublethal effects in bees (Belzunces et al., 2012; Desneux et al., 2007). For instance, the pyrethroid cypermethrin affects insect sodium channels

(Kadala et al., 2014) and has been demonstrated to affect longevity (Muljar et al., 2012) and respiratory patterns (Mänd and Karise, 2015) in bees. Various insecticides taken up in sublethal concentrations are considered to have negative effects on learning, foraging behaviour, or development of individual honey bees (Aliouane et al., 2009; Dai et al., 2010; Urlacher et al., 2016; Williamson et al., 2013; Wu et al., 2011). A direct reduction on egg laying of bumble bee queens was additionally reported from the sublethal effects of the new class of insecticide, sulfoxaflor (Siviter et al., 2018, 2020).

1.2.2. Herbicides and fungicides

Although insecticides are used globally, they are outweighed in terms of sales, quantity applied (Eurostat, 2022; Jess et al., 2018), and market value (US EPA, 2017), by fungicides and herbicides. Herbicides, which target plants, and fungicides, which target fungal diseases, are commonly used in agriculture, in areas where bees and other pollinators are active (Bernauer et al., 2015; Cullen et al., 2019; Muratet and Fontaine, 2015; Meftaul et al., 2019). Yet, since they are not designed to target insects, little is known as to whether their residues in the environment pose an actual risk to bees and other insect pollinators (Cullen et al., 2019; Rondeau and Raine, 2022). Recently, concerns were raised about the impact of herbicides and fungicides on bee health either at single sublethal doses or in combination with other active substances (Tosi et al., 2022).

Herbicides are chemical agents which are used to kill or inhibit unwanted plants or "weeds" (Oliveira et al., 2020; Thiour-Mauprivez et al., 2019), either by killing only the plant parts contacted by the chemical agent, or by systemic action, which allows them to be absorbed through the roots or leaves of the plant and then moved to a different location within the plant (McGinley et al., 2022). Herbicides are the most utilized products in agriculture followed by fungicides and insecticides (Sharma et al., 2019; López-Ballesteros et al., 2022). Herbicides represent more than 40% of crop pesticides applied worldwide (Sharma et al., 2019). Globally, the quantity of herbicides used is twice that of fungicides and almost four times that of insecticides (Zhang et al., 2019), while in the EU the quantity of fungicides and herbicides used are similar and both more than double that of insecticides (FAOSTAT, 2022). Herbicides can affect bees indirectly by reducing the diversity and abundance of flowering plants, which are important food resources for insect pollinators (Bretagnolle and Gaba, 2015), and potentially exacerbating the impacts of other stressors (Freemark and Boutin, 1995; Bohnenblust et al., 2016). Furthermore, emerging evidence has suggested that herbicides can harm bees directly through oral or contact exposure (Sharma et al., 2018; Straw et al, 2021; Weidenmüller et al.,

2022). Although herbicides target physiological pathways specific to plants, they have the potential to interfere with metabolic and reproductive processes in pollinators as well. For instance, paraquat is highly toxic to honey bees when applied topically (Moffett et al., 1972) and 2,4-D and 2,4-trichlorophenoxyacetic acid can negatively influence brood development (Moffett et al., 1972). Glyphosate is the most widely used herbicide in the biggest part of the world, and while it is considered to have low toxicity to bees from a regulatory perspective, evidence shows a variety of negative effects on bees (Abraham et al., 2018; US EPA, 2017; Balbuena et al., 2015; Liao et al., 2017) and a recent meta-analysis linked its use with increased mortality of bees (Siviter et al., 2021). Glyphosate targets a pathway (5- enolpyruvylshikimate-3-phosphate synthase) found not only in plants but in certain bacteria of the gut microbiome of bees, and exposure to glyphosate under laboratory conditions could affect gut microbiota of bees with consequences for both nutrition and susceptibility to pathogens (Cullen et al., 2023; Gabriel and Tscharncke, 2017; Motta et al., 2018), impair factors such as bee navigation, learning and larval development (Balbuena et al., 2015; Dai et al., 2018b; Mengoni Goñalons and Farina, 2018; Vázquez et al., 2018), and alter various aspects of behaviour and physiology (Herbert et al., 2014; Helmer et al., 2014; Weidenmüller et al., 2022).

Approximately four hundred thousand tons of fungicides are applied globally, which represents 17.5% of global pesticide applications (Sharma et al., 2019) and fungicide sales exceed 40% of the total pesticide sales in the EU (Eurostat, 2022). Since they are regarded as safer than insecticides for bees (Heller et al., 2020; Tamburini et al., 2021), fungicides can be applied even when insect attractive crops are in bloom (Favaro et al., 2019; Gierer et al., 2019; Kopit and Pitts-Singer, 2018). Hence, bees foraging in agricultural settings are more likely to encounter fungicides commonly used to control diseases in crops (McArt et al., 2017a; Mullin et al., 2010; Pettis et al., 2013; Rondeau et al., 2022; Rondeau and Raine, 2022). Recent evidence shows that more fungicides than herbicides, and very few insecticides were recorded in insects (Brühl et al., 2021). Indirect effects of fungicides on pollinators include alteration of patch-level floral displays in some plant species due to impacts on mycorrhizae, which can in turn alter pollinator visitation (Cahill Jr et al., 2008). While fungicides are usually not considered acutely toxic to bees (Johnson, 2015), some active substances with low toxicity may pose chronic risks to bees if they are repeatedly present in the environment throughout the season (Long and Krupke, 2016; Tsvetkov et al., 2017). For example, the fungicide boscalid can lead to chronic and cumulative toxicity in adult honey bees (Simon-Delso et al., 2018), possibly by reducing their foraging efficiency (Liao et al., 2019) or by disrupting bacterial metabolism in honey bees (Paris et al., 2020), while sublethal co-exposure with other fungicides may cause significant stress to honey

bees associated with colony decline (Simon-Delso et al., 2014). A low toxicity fungicide was found to negatively affect male reproductive success by altering behavioural and chemical cues in solitary bees (Boff et al., 2022). Research suggests that fungicides may affect food consumption, metabolism, and the immune response (Cizelj et al., 2016; Liao et al., 2017; Mao et al., 2017), and there is increasing evidence of both lethal and sublethal impacts following exposure to some widely used fungicides from studies of both honey bees and wild bees (Belsky and Joshi, 2020; Cullen et al., 2019). In bumble bees, fungicides negatively affected bumble bee reproduction (Bernauer et al., 2015; McArt et al., 2017b), and disrupted floral odour learning and recognition (David et al., 2022). At the individual level, effects of various fungicides induced increased mortality of larvae and adults in both honey bees (Dai et al., 2018a; Domingues et al., 2017; Fisher et al., 2017; Ladurner et al., 2005; Mussen et al., 2004) and solitary bees (Ladurner et al., 2005, 2008), disruptions of feeding behaviour in honey bees (DeGrandi-Hoffman et al., 2015) and nest recognition in solitary bees (Artz and Pitts-Singer, 2015), and a reduction of sperm viability in honey bee drones (Fisher and Rangel, 2018). Fungicides have also been found to indirectly effect bee health mediated through their impacts on beneficial bee microbes that generally work to inhibit bee diseases (Yoder et al., 2011, 2012; McFrederick et al., 2014), e.g., on bee gut microbiota (Steffan et al., 2017) and fungi in stored pollen (Yoder et al., 2013). Exposure to fungicides can make various bee species susceptible to pathogens (McArt et al., 2017b) and viruses (DeGrandi-Hoffman et al., 2015), increasing their mortality rates (Krichilsky et al., 2021). The application of fungicides when bees are present has complex and potentially detrimental effects on both crop pollination (Jaffe et al., 2019) and associated bees (Albert, 2018; Mullin et al., 2010; Goulson et al., 2015).

1.2.3. Bee exposure to compound mixtures

In an agricultural environment, bees are likely to be exposed to multiple contaminants simultaneously (Rondeau and Raine, 2022; Sánchez-Bayo and Goka, 2014; Traynor et al., 2016), causing concerns over negative synergistic effects among them (Fisher II et al., 2017; Kiljanek et al., 2017; Siviter et al., 2021; Traynor et al., 2016). Exposure to two fungicides simultaneously through application of a commercial product (pyraclostrobin and boscalid) changed worker foraging behaviour and reduced colony population size by lowering worker longevity (Fisher et al., 2021a), compromised feeding activity and subsequently the immune function due to lack of nutrition (DeGrandi-Hoffman et al., 2015), reduced mitochondrial function (Campbell et al., 2016), impaired foraging abilities and substantially weakened colony health (DesJardins et al., 2021), inhibited respiration in fungal cells (Earley et al., 2012), reduced thorax mass (Glass et al., 2021) and honey bee queen emergence (DeGrandi-Hoffman et al., 2013), and reduced adult

worker population (Fisher et al., 2021b). It was previously reported that fungicide-fungicide combinations can be just as harmful as fungicide-insecticide interactions and that herbicide and other groups can be more harmful than fungicides and showed similar interactive effects (Iwasaki et al., 2021). The survival of honey bees was significantly reduced after exposure to three pesticides representing the three main pesticide groups (insecticide imidacloprid, fungicide difenoconazole and herbicide glyphosate) (Almasri et al., 2020). Several pesticides, mainly fungicides, that alone are considered not or slightly toxic for bees, in combination with low residues of other compounds, can produce adverse effects (Iwasaki et al., 2021; Tadei et al., 2019; van der Sluijs et al., 2015; Wade et al., 2019). For example, exposure to sterol-biosynthesis-inhibitors (SBI) (including the azoles) can have unexpected synergistic interactions with other pesticides negatively impacting bees (Belden, 2022; Sgolastra et al., 2017, 2018; Siviter et al., 2021). The primary factor in synergies is the presence of azole fungicides, and the concentration of azole fungicides plays a large role in synergy, especially in combination with neonicotinoid insecticides (Belden, 2022; Iwasaki and Hogendoorn, 2021). Specifically, this amalgam may amplify the adverse effects of neonicotinoid insecticides or acaricides on pollinators (Biddinger et al., 2013; Iwasa et al., 2004; Martinello et al., 2017; McArt et al., 2017a; Peghaire et al., 2020; Pilling and Jepson, 1993; Sgolastra et al., 2017, 2018; Spurgeon et al., 2016; Tosi and Nieh, 2019; Tsvetkov et al., 2017; Wernecke et al., 2019; Willow et al., 2019; Zhu et al., 2014) and increase the susceptibility of honey bees and bumble bees to pathogens (Glavinic et al., 2019; McArt et al., 2017b; O'Neal et al., 2018; Paris et al., 2020; Pettis et al., 2013; Tesovnik et al., 2020). Investigations of mixtures of some active substances have confirmed joint actions by additive and/or synergistic effects on physiological processes, often increasing the susceptibility to single pesticides in a pesticide mixture (Gill et al., 2012; Pilling et al., 1995). Carnesecchi et al. (2019) reviewed binary mixtures of all types of chemical contaminants and reported that synergism is common, especially with azole fungicide-insecticide combinations, and highlighted that most synergistic effects were observed as interactions between SBI fungicides and insecticide/acaricide, and neonicotinoid insecticides have been co-detected with SBI fungicides in bumble bees (Raimets et al., 2018; Robinson et al., 2017). SBI fungicides can increase the toxicity of neonicotinoid and pyrethroid insecticides by inhibiting the activity of cytochrome P450 enzymes to metabolize these toxic compounds (Iwasa et al., 2004; Johnson et al., 2013; Pilling et al., 1995; Raimets et al., 2018; Sgolastra et al., 2017). Pyrethroid and triazole/imidazole fungicide combinations likewise had consistent negative interactive outcomes (Iwasaki et al., 2021). In honey bees, prochloraz synergized both pyrethroid (Thompson and Wilkins, 2003) and pyrazole (Johnson et al., 2013) insecticides, and

thiamethoxam (a neonicotinoid insecticide) is synergized by both tebuconazole (Thompson et al., 2014a) and boscalid (Tsvetkov et al., 2017). It was found that bees were exposed to pesticides above the established levels of concern and that synergisms between a few local insecticides and landscape-level fungicides reduced wild bee visitation to cucurbit flowers (Bloom et al., 2021). Even wild bees are exposed to a mixture of multiple pesticides when foraging in agricultural landscapes (Botías et al., 2017; David et al., 2016; Rondeau et al., 2022), but knowledge of the potential synergistic effects of pesticides on them is still poorly understood. Considering the numerous potential mixtures that bees are likely to be exposed to in the field, it is concerning that the combined effects of most of these mixtures are currently unknown (Rondeau and Raine, 2022).

The presence of pesticides on farmland could diminish the conservation and restoration of pollinator ecosystem services by impairing bee health and inhibiting service provision. It is important to avoid the prolonged and unsustainable use of pesticides (Cullen et al., 2019), and has therefore been recommended that a broad evidence base needs to be assessed prior to the development of policy and regulation of pesticides new to the market (Siviter et al., 2018). However, as most studies have been carried out in the lab rather than in the field (Cullen et al., 2019), there are some disputes about whether exposures used in these studies reflect normal field exposure scenarios (Carreck and Ratnieks, 2014; Cutler et al., 2014). To shape and direct future pollinator conservation efforts there is a need to identify the main pesticide contamination sources and the routes through which bees are getting exposed (Rondeau and Raine, 2022). To do that, realistic pesticide exposure scenarios for environmental risk assessments regarding bees need to be defined by evaluating the occurrence, frequency, duration, and magnitude of pesticide combinations to which bees are commonly exposed to in agricultural habitats and matrices (Franklin and Raine, 2019).

1.3. European Union pesticide regulation and bee risk assessment

The high frequency of detected residues, diversity, and elevated risk of some pesticides, raise serious concerns about the protection level of current pesticide regulatory systems (Silva, 2022). In European Union (EU), pesticides are regulated by the pesticide Regulation 1107/2009 (European Commission, 2009b) and the Maximum Residue Level Regulation 396/2005 (European Commission, 2005), which aim to safeguard the environment, animal, and human health while preserving the EU standards of a high-quality agriculture. Getting a pesticide onto the EU market is a complex and long process that involves multiple actors: pesticide

manufacturers are responsible for carrying out all the initial required risk assessment for the active substance that includes toxicology and metabolism studies using standardized protocols. They also need to provide data on pesticide fate in the environment, and effects on non-target species and then submit it to the Rapporteur Member State (RMS) (European Commission, 2013c, d). Consequently, EFSA (European Food Safety Authority), guides risk assessment methodologies, performs a peer review of the report submitted by the pesticide companies and writes a conclusion to the European Commission (European Commission, 2022d).

To date and based on a guidance document for terrestrial ecotoxicology published in 2002 (European Commission, 2002), active substances can be approved only if it has been demonstrated that their use in pesticide products under the proposed conditions by the manufacturer will result in negligible exposure of honey bees or has no unacceptable acute or chronic effects on colony survival and development. The bee guidance document proposed by EFSA in 2013 (EFSA, 2013) was never endorsed by most of the Member States, thus, never officially implemented during the assessment of applications for approval or for the regular renewal of approval of active substances. Nevertheless, it was used during the evaluation of the three neonicotinoids clothianidin, imidacloprid and thiamethoxam leading to their outdoor use ban (European Commission, 2022e).

The European Commission by further considering the impacts on active substances to bee pollinators, a goal that is in accordance with the ambitions of the European Green Deal and will also contribute to achieving the objectives of the Farm to Fork and Biodiversity strategies, mandated EFSA to review the 2013 bee guidance document so as to contain the most up-to-date methodologies for conducting risk assessments for honey bees, bumble bees and solitary bees (European Commission, 2022e). EFSA highlighted a lack of sufficiently robust evidence for defining specific protection goals for bumble bees and solitary bees, and any decision made on the current knowledge may require revision considering new data and tools availability (EFSA et al., 2022). This resulted in the EU Commission's proposal to proceed with an undefined threshold of acceptable effects approach for non-*Apis* species (European commission, 2022g). One of the disadvantages is that trigger values are not yet defined for non-*Apis* species (EFSA et al., 2022), as non-*Apis* data are limited and only based on a single bumble bee and solitary bee species (*B. terrestris* and *O. bicornis* respectively). Moreover, this approach will result in a different lower tier risk assessment approach than the one proposed for honey bees, adding more complexity to the risk assessment and decision-making processes. At higher tier level, only a general protection goal could be implemented, constituting harmonised assessment between different substances more challenging (EFSA et al., 2022).

Most active substances are still not tested on wild bee species so their impacts are not known (Cullen et al., 2019), and pesticide labelling only specifies measures to reduce their impact on honey bees (Mottershead and Underwood, 2020). Although honey bees are important pollinators worldwide (Kleijn et al., 2015) and convenient to handle under experimental conditions (Topping et al., 2021), *A. mellifera* is only one of approximately 20,000 species of bees globally (Michener, 2007). Thus, using the honey bee to assess the ecological risk of pesticides and inform regulatory decisions that will represent all bee species is problematic in many aspects (Franklin and Raine, 2019; Topping et al., 2021). Honey bees differ substantially with regards to their eco-physiological and life history traits compared to most other bee species and more/different exposure routes to pesticides have been identified for several non-*Apis* bee species (Boyle et al., 2019; Franklin and Raine, 2019; Sgolastra et al., 2019). Evidence shows that wild bee species may be more sensitive to pesticides than honey bees (Arena and Sgolastra, 2014; Hayward et al., 2019; Mundy-Heisz et al., 2022). In fact, the various bee species differ in terms of their responses to insecticides in both lab and field-based studies (Arena and Sgolastra, 2014; Bloom et al., 2021; Cresswell et al., 2012; Piironen and Goulson, 2016; Rundlöf et al., 2015; Woodcock et al., 2017), and this could be the case for other pesticide classes, like fungicides and herbicides (Cullen et al., 2019). Moreover, when evaluating various bee species for pesticide residues, it was found that while honey bees and wild bees contained a similar number and concentration of pesticides overall, pesticide mixtures varied by bee type (Ward et al., 2022).

Since honey bees live in large perennial colonies, they are more likely to buffer the side effects of pesticides as opposed to bumble bees and solitary bees (Franklin and Raine, 2019; Henry et al., 2015; Sgolastra et al., 2019), with bumble bees going through a solitary phase from autumn to spring and solitary bees constructing and provisioning their own nest as individuals (Danforth et al., 2019). Current pesticide risk assessments for bees lack the ability to represent acute and chronic exposure to bees from soil and other nesting substrates (Boyle et al., 2019; Franklin and Raine, 2019; Rondeau and Raine, 2022; Sgolastra et al., 2019), while there are big uncertainties regarding the exact levels of pesticides and the compound types that the various bee species are getting exposed to. Even though there are some recent studies that have evaluated the pesticide residues in soil with respect to bee exposure (Rondeau et al., 2022; Willis Chan et al., 2019), sublethal impacts on honey bees and wild bees may not be assessed (Sánchez-Bayo and Tennekes, 2017; Siviter and Muth, 2020), adjuvants and inert ingredients are understudied (Cullen et al., 2019; Straw et al., 2022) and not included in the risk assessments (Mesnage and Antoniou, 2018) despite their toxicological risk (Werneck et al., 2022), and the same applies

for the various pesticide compound mixtures that bees may encounter while foraging in the agricultural environment (Cullen et al., 2019; Rondeau and Raine, 2022; Sgolastra et al., 2020; Siviter et al., 2021). It is important to determine the effects of commercially available pesticides on multiple bee species using methodology that accurately reflects all possible exposure routes (Cullen et al., 2019), by diversifying the type of research (i.e., exposure route, study species and type of exposure) and the range of compounds investigated.

1.4. Importance of exposure through pollen and nectar

Exposure of bees to environmental contaminants can occur either orally, by contact or, to a lesser extent, through the inhalation of volatilized particles in the air (Stanley and Preetha, 2018). The likelihood of exposure to residues for bees is closely related to how and when a pesticide is applied, the matrix and other chemicals it interacts with, and its physiochemical properties and persistence in the environment (Gierer et al., 2019). When pesticides are applied on crops, droplets from spraying or dust created during sowing, can drift from the application site as they are dispersed by wind and can fall directly on bees that are foraging or flying in the vicinity of the treated crop (Sánchez-Bayo and Goka, 2016). Drift can also contaminate nearby foraging and nesting resources, such as wildflowers (Botías et al., 2015; David et al., 2016; Sánchez-Bayo and Goka, 2014, 2016; Traynor et al., 2016), soil (Willis Chan et al., 2019; Rondeau et al., 2022), and water (McCune et al., 2021; Samson-Robert et al., 2014; Zubrod et al., 2019). Most of seed-coated systemic pesticides leach into the soil (up to 98%, Goulson, 2013), where water can distribute it across the field and even into the margins, where the chemical can be taken up by non-target habitat vegetation (Botías et al., 2015; Mogren and Lundgren, 2016). This may lead to both the ingestion of contaminated pollen and nectar or direct contact exposure for bees with contaminated surfaces (Gradish et al., 2019; Kopit and Pitts-Singer, 2018; Sgolastra et al., 2019). Furthermore, due to the persistence of pesticides in soils (e.g., some neonicotinoid compounds, Lewis et al., 2016), subsequent crops and non-target plants can still absorb residues several months after the pesticides were introduced. Because of this, landscapes could produce contaminated pollen and nectar food resources for lengthy periods, such as Wintermantel et al. (2020) demonstrated by finding imidacloprid in oilseed rape crop nectar five years after it had been banned from use on outdoor flowering crops. The uptake of pesticides by foraging bees into their hives/nests and the contamination of food storage might cause long-term effects on colony development (Boyle et al., 2019; Mullin et al., 2010). Systemic herbicides and fungicides could also pose a risk to bees due to their varying persistence period in the environment, leading to multiple pesticide exposure even when they are not applied in close temporal proximity to when the bees are foraging on the crops, however there is no

complete understanding of their relative risks (Cullen et al., 2019). Significant variability of pesticide residue levels can occur, which depends on several factors including crop type and application method (Gierer et al., 2019) and residues may degrade over time via exposure to environmental conditions, metabolic activity of microorganisms in soil and on plants, etc. (Katagi, 2020).

Compared to other exposure routes (e.g., air particles, soil, guttation fluid etc), dietary exposure through consumption of pollen and nectar is thought to be a significant exposure route, very relevant across all bee species (Bireley et al., 2019; Boyle et al., 2019; Cham et al., 2019; Hinarejos et al., 2019; Sgolastra et al., 2019; US EPA et al., 2014). Most female adult bees visit flowers to collect pollen to provision their larvae and consume both nectar and pollen themselves directly from flowers (Boyle et al., 2019). Both pollen and nectar are nutritional sources of several amino acids, minerals, micronutrients, and secondary metabolites (Palmer-Young et al., 2019; Wright et al., 2018). Pollen is primarily required to raise brood and contributes to sustained colony population growth throughout the growing season in honey bees (Smart et al., 2016). Pollen specifically, has been indicated as the best matrix for assessment of the presence of environmental pesticide residues, as it is easy to collect and is frequently contaminated (Chauzat et al., 2006, 2011). Bee pollen is a very complex matrix that is a particular analytical challenge for pesticide residue analysis (López-Fernández et al., 2015), as it is composed of proteins, lipids, sugars, fibre, mineral salts, amino acids, phenolic compounds, and vitamins (Campos et al., 2008). A key limiting factor for evaluating concentrations of pesticides in nectar or pollen is the difficulty in obtaining sufficient volume to conduct the residue analyses (Cowles and Eitzer, 2017), and their production and quantity is strongly related to environmental conditions during the time of flowering and to soil and plant type (Knaebe et al., 2018). A minimum of ~1000 flowers need to be collected to extract enough pollen and nectar for analysis (Botías et al., 2015). Residue levels in nectar were found to be lower than the respective levels in pollen and strong positive correlation was observed between nectar and pollen residues in oilseed rape (Kyriakopoulou et al., 2017), but it is unclear whether this relationship applies for all compound types, and for wild plant residues.

To date, most research on the lethal and sublethal effects of pesticides on pollinators focuses on the consumption of contaminated pollen and nectar (Franklin and Raine, 2019; Sgolastra et al., 2019), thus it is essential to summarise the existing information on pesticide residues levels in pollen and nectar detected up to date, and to provide more information on the field realistic levels of pesticides that bees may encounter in agricultural fields while foraging for pollen and nectar.

1.5. Arable crops in Ireland and most used pesticides

Currently, global pesticide use exceeds 2.5 million tonnes per year (FAOSTAT, 2022). Despite their widespread use, pesticide input varies greatly across regions and farming systems. This is due to differences in pest pressure, economic, technological, and regulatory/legislative factors (Delcour et al., 2015; Sharma et al., 2019; Watts, 2019). There are over 1000 different pesticide products used around the world (WHO, 2022), each with a particular behaviour against pests, various environmental fate characteristics (persistence, biodegradability, and accumulation) and different toxicity levels (Kalyabina et al., 2021). The EU Pesticides Database lists more than 1479 active substances, 455 of which have been approved, 942 have not been approved for use in the EU, and the rest are pending for assessment at a European level (European Commission, 2022c).

The Irish landscape is dominated by agriculture (65% of the total land area), comprising mainly of grass lands, and at a lesser extent, arable crops (DAFM, 2022). In terms of land area, oilseed rape is one of the most abundant entomophilous crops in Ireland, accounting for 9,200 hectares in 2019, while beans are considered the second most abundant pollinator attractive crop, with 8,100 hectares (CSO of Ireland, 2020). In agricultural landscapes, mass flowering crops such as oilseed serve as a valuable nectar and pollen source for honey bees and bumble bees (Rollin et al., 2013; Westphal et al., 2006, 2009). Oilseed rape (*Brassica napus* L.) has bright yellow flowers with open petal arrangements allowing easy access to copious amounts of pollen and nectar (Kunin, 1993; Phillips et al., 2018; Stanley et al., 2013). Plant species of the Fabaceae family, such as beans, are considered major pollen source for most bumble bee species (Mottershead and Underwood, 2020), as they generally prefer protein-rich pollen (Hanley et al., 2008; Leonhardt and Blüthgen, 2012; Vaudo et al., 2015). It has been suggested that mainly long-tongued bumble bees can access nectar from bean flowers (*Vicia faba* L.) due to the floral anatomy (Free, 1993), but visitation by honey bees (Burns and Stanley, 2023; Kendall and Smith, 1975), solitary bees (Tasei, 1976) and other short-tongued bumble bee species (Garratt et al., 2014; Burns and Stanley, 2023) has been observed, although raiding behaviour (nectar-robbing) by some bumble bee species and honey bees is common (Aouar-Sadli et al., 2008, Benachour et al., 2007, Tasei, 1976).

In Ireland, most pesticides are applied directly to crops as a foliar spray (López-Ballesteros et al., 2022). Herbicides account for 76% of the total pesticides placed in the market in Ireland in 2021, followed by fungicides (14%) and insecticides (0.3%) (DAFM, 2021). A total of 120 active substances were recorded in use for arable crops in the latest pesticide usage report for arable

crops in Ireland (DAFM, 2016). The herbicide glyphosate and the fungicide prothioconazole were the most applied active substances on oilseed rape crops, ranked by treated area, while for beans the fungicide boscalid prevailed (DAFM, 2016). The herbicide fluroxypyr was among the most applied herbicides in cereal crops used in rotation with oilseed rape and bean crops and azoxystrobin is a fungicide among the most extensively applied on arable crops in Ireland (DAFM, 2016). Of the neonicotinoid compounds, acetamiprid and thiacloprid were applied only in potato crops (DAFM, 2016). In the present thesis, I chose to evaluate these compounds, along with aminomethylphosphonic acid (AMPA), the main metabolite of glyphosate, and the five neonicotinoid compounds clothianidin, imidacloprid, thiacloprid, and thiamethoxam (Figure 1.1).

1.6. Research objectives and thesis outline

This PhD research aims to supplement the discussion on pesticide risks to bees and tries to expand the knowledge on the field-realistic contamination levels of pollen and nectar with pesticides. In this thesis, particular attention is given to the fate of pesticide compounds and their presence in pollen and nectar of both crops and wildflowers, and bee corbicular pollen, while trying to define the exact mixtures of pesticide residues that bees are likely to encounter while foraging in an agricultural environment, and consequently evaluate the hazard that these residues might pose to bees. Particular emphasis is given in the evaluation of the most widespread used herbicide globally, glyphosate and its metabolite AMPA, since those results could contribute to the ongoing debate on the re-approval of glyphosate use in the EU.

This thesis includes six chapters. Chapter 1 gives a general introduction to pesticides and their impact on bees, the respective EU regulatory framework, and the knowledge gaps with regards to nectar and pollen contamination; Chapters 2 to 5 address the main PhD research objectives, and Chapter 6 discusses the main findings and future implications of the work.

The thesis research objectives as addressed in each chapter are:

Chapter 1 sets the context of the thesis, presenting the importance of bees in pollination and worldwide economy, the potential contribution of various pesticide types in bee declines, identifies limitations of the current pesticide regulatory system on risk assessment for bees in EU, highlights the importance of pollen and nectar as exposure routes to pesticides, and finally gives an overview of the pesticide usage in Ireland.

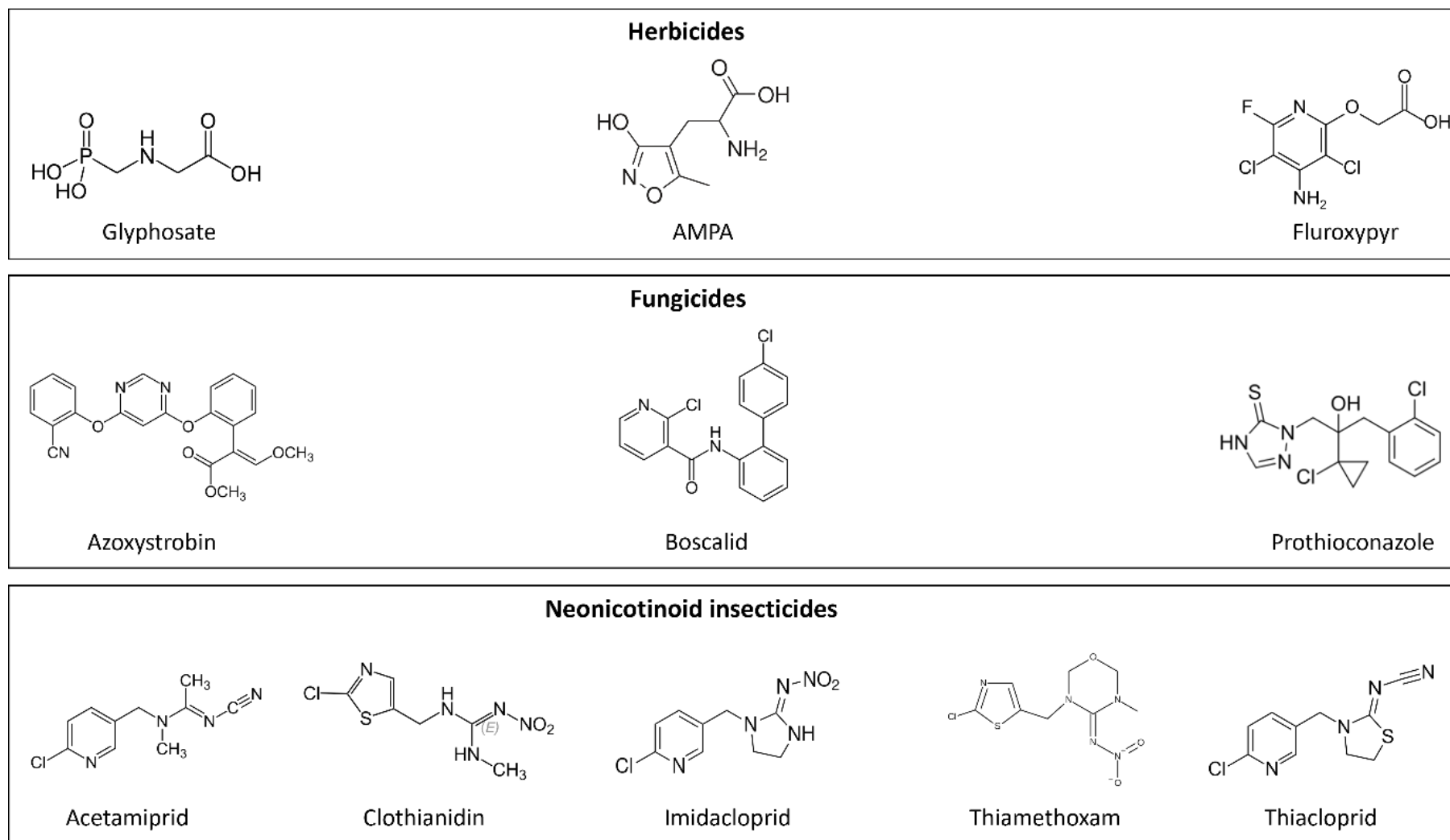


Figure 1.1. Chemical structure of the ten pesticide compounds and one metabolite (AMPA) evaluated in the present research.

Chapter 2 summarizes the current worldwide knowledge on pesticide residue levels in pollen and nectar directly collected from plants (rather than from bee collected samples) and identifies the knowledge gaps on pesticide residue research in nectar and pollen. It is the first systematic literature review that summarises and critically evaluates pesticide residue levels and occurrence in nectar and pollen directly collected from plants. This chapter includes details on the individual compounds and their mixtures detected in pollen and nectar, their contamination levels and frequency of detection and whether they could pose a hazard for bees. Comparisons between concentrations of compounds measured in cultivated versus wild plants are made and future research directions are proposed.

Chapter 3 compares the mixtures and levels of pesticide residues in pollen and nectar of crops and wild plants growing in the margins of the crops. Pesticide detection patterns are presented, and the most common compound mixtures are identified. Continuous monitoring of neonicotinoid presence and fate in the field environment is strongly recommended.

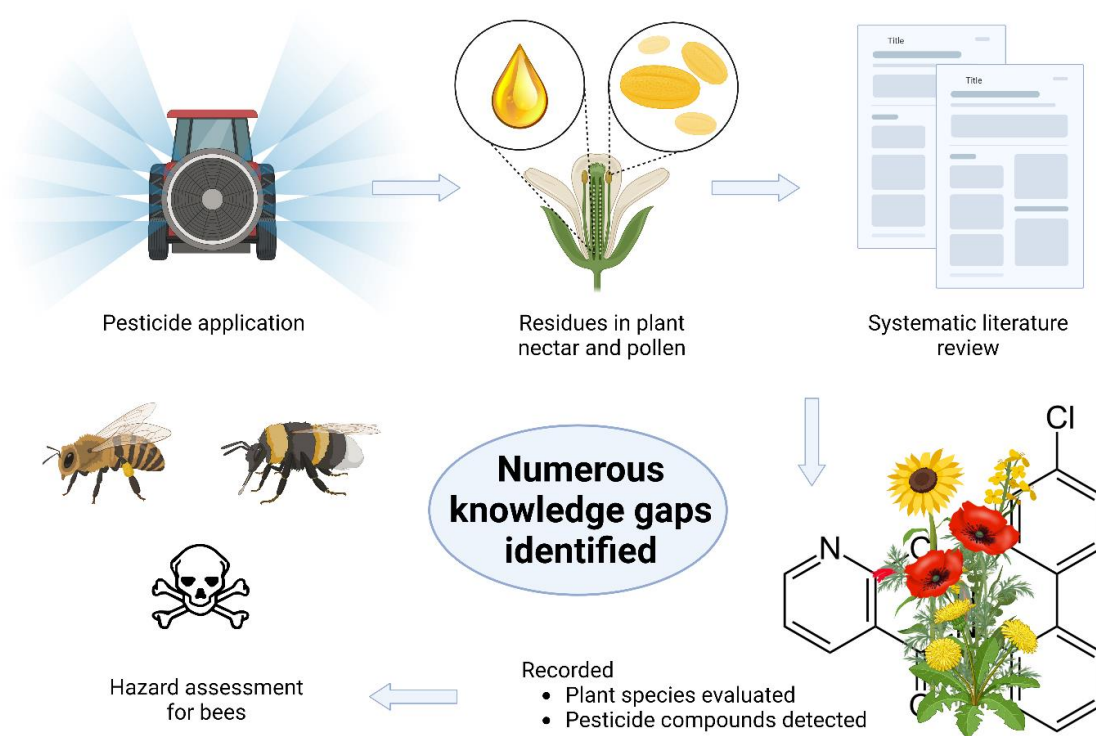
Chapter 4 compares the mixtures and levels of pesticide residues in pollen of different crops and corbicular pollen from honey bees and bumble bees foraging simultaneously under the same agricultural conditions. Pesticide contamination patterns are discussed, and a comparison is made with similar findings of other studies. It is argued whether the different bee species are exposed to pesticides in different ways.

Chapter 5 determines the occurrence and levels of glyphosate and its main metabolite AMPA in pollen and nectar of both crops and wild plants, as well as honey bee and bumble bee collected pollen under realistic agricultural conditions. This evaluation of glyphosate residues in an agricultural environment is performed for the first time. Potential off-target contamination pathways of glyphosate are discussed, and concerns are raised for possible implications of glyphosate residues detected in nectar, connected to bee and human health.

Chapter 6 summarizes the major outcomes of this PhD research and the respective implications on pesticide residue bee exposure and risk assessment, and pesticide related EU Strategies. This chapter concludes with recommendations on these areas and directions for future work.

Chapter 2

Plant protection product residues in plant pollen and nectar: a review of current knowledge



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2. Plant protection product residues in plant pollen and nectar: a review of current knowledge

2.1. Abstract

Exposure to Plant Protection Products, PPPs, (fungicides, herbicides and insecticides) is a significant stressor for bees and other pollinators and has recently been the focus of intensive debate and research. Specifically, exposure through contaminated pollen and nectar is considered pivotal, as it presents the highest risk of PPP exposure across all bee species. However, the actual risk that multiple PPP residues might pose to non-target species is difficult to assess due to the lack of clear evidence of their actual concentrations. To consolidate the existing knowledge of field-realistic residues detected in pollen and nectar directly collected from plants, we performed a systematic literature review of studies over the past 50 years (1968-2018). We found that pollen was the matrix most frequently evaluated and, of the compounds investigated, the majority were detected in pollen samples. Although the overall most studied category of PPPs were the neonicotinoid insecticides, the compounds with the highest median concentrations of residues in pollen were: the broad spectrum carbamate carbofuran (1,400 ng/g), the fungicide and nematocide iprodione (524 ng/g), and the organophosphate insecticide dimethoate (500 ng/g). In nectar, the highest median concentration of PPP residues detected were dimethoate (1,595 ng/g), chlorothalonil (76 ng/g), and the insecticide phorate (53.5 ng/g). Strong positive correlation was observed between neonicotinoid residues in pollen and nectar of cultivated plant species. The maximum concentrations of several compounds detected in nectar and pollen were estimated to exceed the LD_{50s} for honey bees, bumble bees and four solitary bee species, by several orders of magnitude. However, there is a paucity of information for the biggest part of the world and there is an urgent need to expand the range of compounds evaluated in PPP studies.

2.2. Introduction

High overwintering losses of honey bee colonies and declines in populations of other insect pollinator species in both Europe and North America (Gierer et al., 2019; Lee et al., 2015; Ollerton, 2017; Potts et al., 2010b; Seitz et al., 2016) have raised public and political concerns about the contribution of Plant Protection Products (PPPs) to bee decline (Cressey, 2015; IPBES, 2016).

While there are numerous factors contributing to pollinator decline, the intensification of agriculture over the past six decades, and with it the widespread use of synthetic PPPs, is considered to be a major driver of insect losses (Arena and Sgolastra, 2014; Goulson et al., 2015; Dudley and Alexander, 2017; Rundlöf et al., 2015; Sánchez-Bayo and Wyckhuys, 2019; Williams et al., 2015). PPPs are very widely used (Dudley and Alexander, 2017), and can be very persistent in the environment (Casado et al., 2019; Silva et al., 2019), potentially exposing non-target organisms to mixtures of toxic residues (Botías et al., 2016; Gavrilescu, 2005; Looser et al., 2000). For bees, which have been the focus of widespread concern, PPP exposure routes include particles in the air (dust and spray), nectar, pollen, mud/soil, wax, water, guttation fluid, plant surfaces, and propolis/resin (Boyle et al., 2019). In comparison with other exposure routes (e.g., air particles, soil, guttation fluid etc), dietary exposure through consumption of pollen and nectar is thought to be a significant exposure route, posing highest risk across all bee species (Bireley et al., 2019; Boyle et al., 2019; Cham et al., 2019; Hinarejos et al., 2019; Sgolastra et al., 2019; US EPA et al., 2014).

If plants are flowering at the time of application, nectar and pollen can become contaminated with PPPs directly, although most practitioners avoid spraying insect-pollinated crops whilst the crop is in flower (Larson et al., 2013, 2015). Due to the systemic nature of some PPPs, the active substance may also be translocated from the soil through the plant tissues, contaminating pollen and nectar (Botías et al., 2015; Cowles and Eitzer, 2017; Stoner and Eitzer, 2012). Since bees bring pollen and nectar back into the nest (Poquet et al., 2016; Sanchez-Bayo and Goka, 2014; Sgolastra et al., 2019), this can create an unintended accumulation of PPP mixtures in stored nectar and pollen (Boyle et al., 2019), and, in the case of honey bees, in other hive materials (e.g., wax etc) (Benuszek et al., 2017). Once this happens, not only the forager bees, but also the brood and, in social species, the rest of the colony (brood, nurse bees, queen, drones) are at risk of exposure to these chemicals (Prado et al., 2019). Recently published analyses of pollen from managed honey bees located near agricultural sites demonstrated that many chemicals (including insecticides, miticides, fungicides and herbicides) were detectable in

various hive matrices (Johnson et al., 2012; Lambert et al., 2013; Mullin et al., 2010; Wu et al., 2011). In addition to the active substances, bees may also be exposed to additives used in PPP formulations, and these have also been detected in pollen and honey with the potential to interact with PPPs and increase toxic effects (Mullin et al., 2015). Hence, many bee species are likely to be chronically exposed to mixtures of multiple PPPs, throughout their development and adult life, particularly when residing in intensively managed arable and horticultural landscapes (Roszko et al., 2016). The impact of PPP exposure on bees has been tested in numerous lab- and field-based experiments in recent years (Arena and Sgolastra, 2014; Cedergreen 2014; Goulson et al., 2015; James and Xu, 2012; O'Neil et al., 2018; Sánchez-Bayo and Goka, 2014; Wood & Goulson, 2017), but these experiments can be limited by lack of knowledge of field-realistic doses of these compounds (Lydy et al., 2004; Pohorecka et al., 2012; Siviter et al., 2018).

Field exposure to PPPs from ingesting contaminated nectar and pollen is not only likely to vary across crops and seasons, but also between wild bees and managed social bees (Rundlöf et al., 2015). For example, bumble bees seem to use and benefit similarly from the resources available in a farmland (Wood et al., 2015b), while bees from other genera, especially solitary bees, may show more variation in their foraging choices (Wood et al., 2016), and thus, a variable range of levels of exposure to agrochemicals (Botías et al., 2017). Moreover, while honey bees tend to select nectar sources that provide the greatest sugar reward, this may not always be the case with solitary bees (Boyle et al., 2019). Big differences can be also observed in the consistency and constituents of the food mass across bee species, as they may handle their food in different ways (e.g., in social bees, pollen is processed by hypopharyngeal glandular secretions before storage) (Nixon and Ribbands, 1952), store for different amounts of time, and some have particular dietary requirements, and thus forage on specific plant species (Benton, 2017).

However, most female adult bees visit flowers to collect pollen to provision their larvae and consume both nectar and pollen themselves directly from flowers. Thus, it seems that one way to measure the field-realistic exposure of bees to PPP residues is to analyse pollen and nectar directly from plants, and quantify residue concentrations (Stoner and Eitzer, 2012). Determining the quantity, distribution and prevalence of systemic PPP residues present in vegetation is highly relevant for both agricultural management and biodiversity conservation, since establishing the limits of field-realistic exposure is essential to risk assessment for a range of taxa (Botías et al., 2016). Thus, obtaining more information on what constitutes field realistic exposure is vital.

In order to obtain reliable PPP residue data for pollen and nectar, expensive and practically challenging residue studies need to be performed (Gierer et al., 2019). The key limiting factor

for evaluating concentrations of PPPs in nectar or pollen is the difficulty in obtaining sufficient quantities to conduct the residue analyses (Cowles and Eitzer, 2017). Since nectar is a much more difficult matrix to collect for many plant species, it is useful to evaluate whether there is a relationship between the two matrices, and whether the values of pollen residues can be used as a predictor for the concentrations found in nectar.

In the present review, we evaluate the current knowledge of field realistic residues of PPPs found in nectar and pollen directly collected from plant species, outline the existing knowledge gaps and highlight the future research needs for residue studies and pollinator risk assessments.

2.3. Materials and methods

2.3.1. Key concepts and research strategy

A systematic review was conducted to address the question: ‘What are the field realistic residues of PPPs that bees are exposed to when foraging on nectar and pollen’? The key elements were: (1) bees as main non-target organism of interest, (2) PPPs as the intervention of interest, (3) pollen and nectar directly collected from plant resources as the main matrices evaluated, and (4) the concentrations of the main active substances and the plant species evaluated as the outcomes of interest. The reason bees were chosen as a species of interest is because when considering non-target impacts of PPP use for pollinators, regulatory agencies around the world are making decisions using data from mainly bee species as the only model insect pollinator (EFSA, 2013; Franklin and Raine, 2019; Uhl and Brühl, 2019). After initial assessment of publications related to bee PPP exposure, keywords were determined and a systematic literature search was carried out in November 2018 using the databases of Web of Science Core Collection and Scopus. The following search terms to locate potential studies on PPP (pesticide) residues and bees were used: (bee* OR *Apis* OR honey bee* OR bumble bee*) AND (pesticide* OR insecticide* OR fungicide* OR herbicide*) AND (residue* OR exposure) AND (pollen OR nectar)/(pollen) AND (nectar). No limitations of dates or language were applied to the search strategy. To be included in this review, papers must have reported primary research, and detected at least one concentration of PPP in pollen or nectar directly collected from a plant species. Papers referring to PPP residues in pollen and nectar that were processed by bees (e.g., pollen collected from pollen traps, stored pollen or nectar etc.) were not included.

2.3.2. Data extraction protocol

Each publication was reviewed and data was extracted and recorded in a main dataset, including: location of study (if this information was lacking, the location of the first author’s

institution was used as a proxy); active substance; time, rate and method of application(s); product name; PPP category (e.g. insecticide, fungicide, herbicide); mode of action (systemic or not); plant taxa (family and scientific name), flowering status during treatment (flowering or not) and state (cultivated or wild); growing and treatment conditions (substrate, indoor/outdoor cultivation); time of collection of matrices (days after treatment); volume of matrices analysed; matrix analysed (pollen, nectar, other); concentration of residues (ng/g); limits of detection (LOD); method of chemical extraction (e.g., QuEChERS etc); analysis method (e.g., UHPLC-MS/MS etc); correlations with other matrices evaluated; and metabolites evaluated. In order to facilitate our analysis, we used a more simplified form of the main dataset. Cultivated plants included (any plant that was part of an actual or artificial cultivation) and wild plants denoted (any plant that was growing naturally in the environment). For each plant species, we recorded its common name, its synonym according to the nomenclature, and its variety (for cultivated plants) (see Appendix A Table A1). The nomenclature of the majority of the plant species was based on Euro+Med PlantBase (2006), and the Plant List database (2013) was used for the varieties of the cultivated plants. As has already been noted by Benuszak et al. (2017), different units are used to express concentrations (i.e., parts per billion, $\mu\text{g}/\text{kg}$ and ng/g) in different studies. We used ng/g for all our calculations and converted other metrics into this unit for analysis. The only exception was the values used for the heatmap (Figure 2.2), where we converted the PPP residues in pg/g , in order to avoid negative values in the Log_{10} scale. In cases where the residue concentration was given as $< \text{LOD}/\text{LOQ}$, we used half of the respective LOD/LOQ values (Dively and Kamel, 2012). The software used for the graph creation was GraphPad Prism 8.

2.3.3. Statistical analysis

The relationship between the concentrations of insecticide residues in nectar and pollen was assessed using a paired Generalized Linear Mixed Model approach. The aim of this model was primarily to assess whether residue concentrations observed in pollen can be used to predict those observed nectar. As residue measurements may differ based on study specific factors, such as treatments applied and laboratory methods used, we first subsetted the data to include only those plant species where both nectar and pollen had been assessed in the same study. Of the 25 studies in our dataset only seven contained residue estimates for both nectar and pollen. Based on these studies, five compounds had sufficient data to be included in further analysis, namely, clothianidin, dinotefuran, imidacloprid, thiacloprid and thiamethoxam (all of which are neonicotinoid insecticides). This resulted in a dataset of 206 residue measurements from both pollen and nectar across 13 plant families. Prior to model fitting, scatterplots of the relationship

between nectar and pollen were visually assessed. These scatterplots showed that both nectar and pollen data were highly skewed, and the variance of both increased with the mean. Furthermore, a linear relationship appeared to exist between the log of these two variables, therefore the natural log of concentration in pollen rather than the raw pollen data was used to predict concentrations in the nectar. The level of residues in the nectar (ng/g) was fitted as the response variable. The natural log of residue concentrations in pollen (ng/g), the compound type, and the 'Status' of the species ('cultivated'/'wild') were fitted as predictor variables (fixed factors). The interaction between the natural log of residues in pollen and 'Status' was included to test for differences in the relationship between residues in nectar and pollen, between wild and cultivated plants. The model accounted for non-independence of data within 'Study' and 'Plant family', by fitting these as non-nested random factors. Models were initially fitted using a Gaussian error distribution, but the plotted model residuals were non-normal and variance was highly heterogeneous. Therefore, the models were refitted using a Gamma error distribution, and a log link function. GLMM's were fitted using the package *glmmTMB* v0.2.3 (Brooks et al., 2017). The amount of variance explained by both fixed and random factors in the model conditional and marginal variance were calculated using the R package *MuMIn* v1.43.6 (Barton, 2019). Conditional variance represents the amount of variation in the response explained by the full model (i.e., both random and fixed effects), and marginal variance represents that which is explained by the fixed factors alone (i.e., here pollen measurements, 'Status' ('cultivated' or 'wild'), and compound type; Nakagawa and Schielzeth, 2013). Analyses were conducted using R version 3.6.1 (R Core Team, 2019).

2.3.4. Calculation of the pollen and nectar Hazard Quotient and Estimated % LD₅₀

In order to put the pesticide residues into a context of risk to bees, we need to have information on both the concentrations of residues detected in pollen and nectar and their respective LD₅₀s. For each compound evaluated in the residue studies reviewed, pollen and nectar Hazard Quotients and Estimated % LD₅₀ were calculated using the methods proposed by Stoner and Eitzer (2013), Sgolastra et al. (2017) and Stoner et al. (2019). Estimating these values is an attempt to illustrate a simplified risk to bees from consuming contaminated food (Traynor et al., 2016). The use of these values is in turn a simplified effort to quantify pesticide exposure, encompassing contact and oral risk from the maximum amount of residues detected in pollen and nectar (Stoner and Eitzer, 2013; Traynor et al., 2016). The LD₅₀ values represent averaged 24, 48 and 72 h adult acute oral and contact toxicities available from the database of the University of Hertfordshire (2019), except for the compound spinosad, where the respective data were retrieved from Miles et al. (2012). In order to evaluate the PPP residues for bee

toxicity, we initially needed to calculate the uptake of each PPP by a bee foraging for pollen and nectar contaminated by the maximum residue of that PPP. Thus, we calculated the Maximum Residue Uptake (MRU) of a certain compound per bee per day (information available for honey bees, bumble bees and solitary bees) (see Appendix A Tables A2.1 and A2.2) and per one foraging bout (information only available for nectar foraging honey bees) (see Appendix A Table A3). The data on the bee daily consumption of nectar and pollen were retrieved from the guidance document on the risk assessment of plant protection products on bees (EFSA, 2013). For the honey bee daily nectar consumption, we used the average consumption value of a forager bee, as they are consuming larger amounts of nectar (Rortais et al., 2005). For the honey bee consumption of nectar per one foraging bout we used the method proposed by Sgolastra et al. (2017). In order to calculate the maximum Hazard Quotient (HQ) for the maximum PPP residues found in pollen and nectar we used the method proposed by Stoner and Eitzer (2013) (see Appendix A Table A3):

$$HQ = \frac{\text{Residue concentration (ng/g)}}{LD_{50} (\mu\text{g/bee})}$$

In addition, the values of the Estimated % LD₅₀ (% LD₅₀) were calculated (see Appendix A Table A3) in order to evaluate how much of the LD₅₀ of a compound would a bee consume per one foraging bout (only for honey bee nectar forager) (see Appendix A Table A3), and per day:

$$\% LD_{50} = \frac{\text{Maximum residue concentration (ng/g)} \times \text{Bee pollen or nectar consumption per day (mg/bee)}}{\text{Oral or Contact LD}_{50} (\mu\text{g/bee})}$$

For compounds where only a lower limit of LD₅₀ was determined, that lower limit was used for calculation (e.g., for LD₅₀ > 100 µg/bee, the value 100 was used) (Stoner et al., 2019). For a clearer presentation of the results, we chose to report the values of Estimated % LD₅₀ in three categories: > 100, 50-100 and < 50%, highlighting the values that exceeded 50% of the bee LD₅₀s.

2.4. Results

2.4.1. General information

The initial database search yielded a total of 5741 articles. After the removal of duplicates (1381 articles), titles and abstracts for 4360 papers were screened for relevance. In the end, only 24 articles referred to measurement of PPP residues in pollen and nectar directly collected from plants. At that stage, four papers (Bailey et al., 2005; Lord et al., 1968; Thompson et al., 2015; Waller and Barker, 1979) were added as they were considered very relevant to the present review and they were cited in the 24 primary studies, but did not appear in the initial database search, bringing the total papers to 28. Among the 28 studies, those of Botías et al. (2016) and

Thompson et al. (2016) reassessed the results of their previous studies (already included in our sample) and thus were removed. Moreover, the study of Cowles and Eitzer (2017) was also not included in the analyses of the data of the present review, since the exact PPP residues were not clearly articulated. Accordingly, 25 papers were included in our analysis.

The first paper was published in 1968 and the majority of papers appeared in the years 2012 and 2015 (Figure 2.1). Seventeen papers evaluated pollen, 16 assessed nectar while both matrices were evaluated in eight studies (Figure 2.1a). Studies were conducted in countries located in North America (48%), Europe (48%) and Asia (4%), while there were no studies from Australia, Africa or South America. The majority of studies were carried out in the United States (n = 11), while the United Kingdom (n = 6), followed by France (n = 4), are the strongest representatives at a European level.

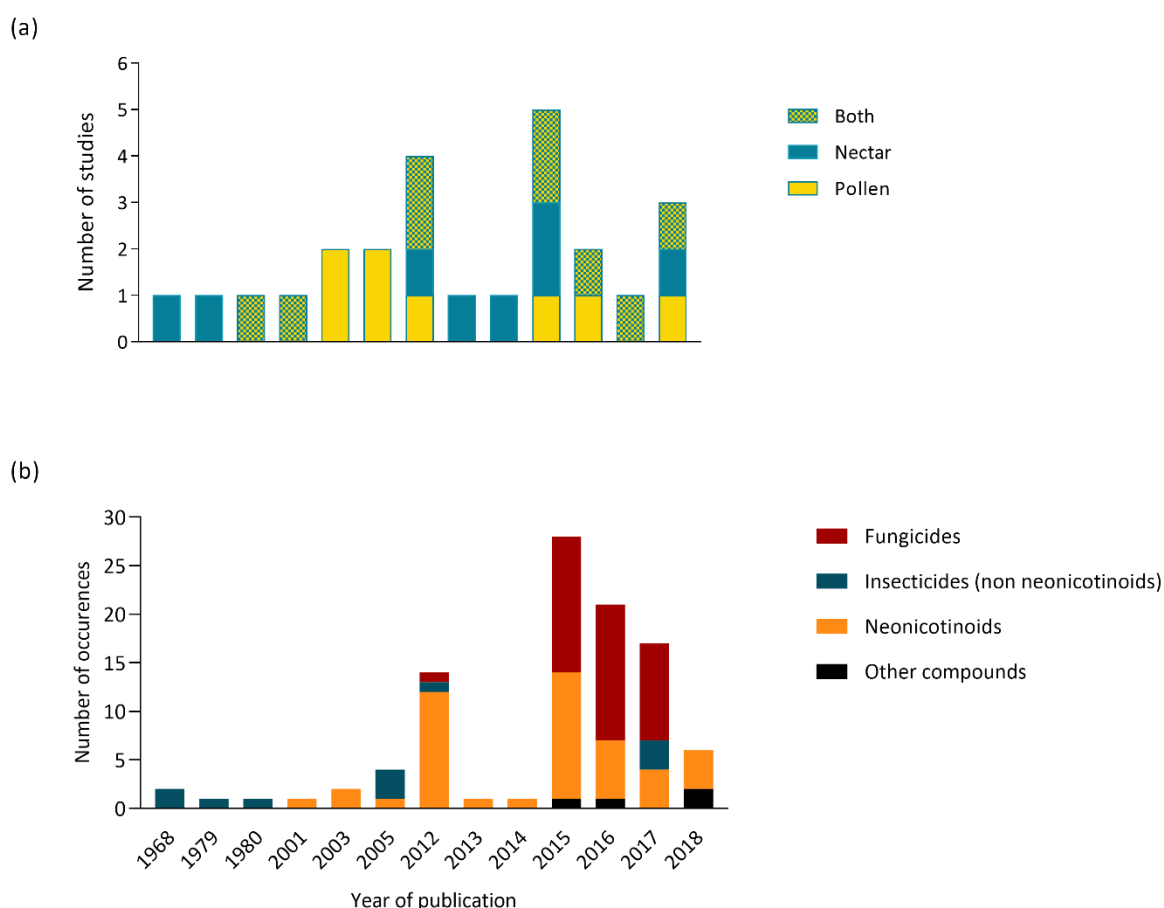


Figure 2.1. a) The number of studies and the matrix analysed each publication year, and b) The number of times a PPP category (fungicides, non-neonicotinoid insecticides, neonicotinoids and others) was evaluated each publication year.

2.4.2. PPPs and Plant Species evaluated

A total of 31 active compounds, two neonicotinoid metabolites and one synergist (a substance that participates in the interaction or cooperation of two or more substances, to produce a combined effect greater than the sum of their separate effects) were identified in pollen and nectar collected directly from plants (Table 2.1). The majority of the 31 compounds were fungicides (n = 17 compounds) and the rest were insecticides (n = 14 compounds), and they were all systemic compounds except for five (fungicides: chlorothalonil and prochloraz; and insecticides: chlorpyrifos, cypermethrin and lambda-cyhalothrin). In terms of mode of action, the majority of the compounds act as acetylcholinesterase and demethylation inhibitors. No studies evaluated the presence of herbicides in pollen and nectar collected from plants. The maximum number of compounds evaluated in a paper was 20 (two studies), and the mean number of compounds evaluated per study was four. The neonicotinoids have been studied since 2001, but fungicides only started to be evaluated from 2012 (Figure 2.1b). The most frequently studied compounds were the insecticides (n = 57 studies), particularly the neonicotinoids (n = 46 studies). Imidacloprid was the most studied compound (n = 17 studies), followed by thiamethoxam (n = 10 studies), and clothianidin (n = 9 studies).

The compounds with the highest median levels of residues in pollen were: the carbamate insecticide carbofuran (1400 ng/g), the dicarboximide fungicide iprodione (524 ng/g), the organophosphate insecticides dimethoate (500 ng/g) and chlorpyrifos (100.5 ng/g), the natural product with insecticide properties spinosad, isolated from the bacterial species *Saccharopolyspora spinosa* (320 ng/g), the chloronitrile fungicide chlorothalonil (265.2 ng/g), and in nectar: dimethoate (1,595 ng/g), chlorothalonil (76 ng/g), the organophosphate insecticide phorate (53.5 ng/g), and the carbamate insecticide oxamyl (35 ng/g) (Table 2.2). For the neonicotinoids, median residues were highest for dinotefuran (34.7 ng/g in pollen, 7 ng/g in nectar), and between 0.01 and 0.08 ng/g for the other compounds in both nectar and pollen (Table 2.2). Metabolites and PPP co-formulants (substances used in commercial formulations in order to optimize the efficacy and the stability of the primary active substances), indicated in Figure 2.1b as "other compounds", have been evaluated since 2015. The majority of PPP reports for residues in pollen and nectar were a result of application of commercial formulations, rather than application of the active substance itself. In those products, the most usually encountered combinations in the formulations were clothianidin with the compound prothioconazole (fungicide), imidacloprid with beta-cyfluthrin (insecticide), spinosad with 1,2-benzisothiazoline-3-one (preservative with fungicidal properties), thiacloprid with deltamethrin (insecticide), and thiamethoxam with metalaxyl-M and fludioxonil (both fungicides). Out of all these co-

formulants, none has been evaluated for plant nectar and pollen residues. Ten of the PPP compounds studied (carbofuran, carbendazim, chlorothalonil, clothianidin, dimethoate, dinotefuran, flusilazole, iprodione, phorate, and thiamethoxam) are not currently approved for use in the European Union - according to Reg. (EC) No 1107/2009 (European Commission, 2009b).

Information on residues of different compounds is of more value when associated with the plant taxa in which they were detected. In total, 94 plant taxa (41 cultivated and 54 wild with each variety of cultivated plant considered a different taxon) from 31 plant families were evaluated for pollen and nectar PPP residues (Table 2.3), and on average, five plant taxa were evaluated in each study. Most taxa evaluated for PPP residues in pollen and/or nectar belonged to Asteraceae (n = 20 taxa). The most studied taxa were cultivated *Zea mays* L. (maize), which was evaluated only for pollen residues (n = 6 studies), and *Brassica napus* L. (oilseed rape), for residues in nectar in five studies and in pollen in three.

In terms of PPP presence, the highest mean numbers of different compounds (reported as > LOD/LOQ) were detected in the families of Poaceae (n = 8), Ericaceae (n = 6), Rosaceae (n = 5) and Brassicaceae/Fabaceae (n = 4). On a species level, in winter *Brassica napus*, 14 different compounds have been detected, in *Fragaria × ananassa* (Duchesne ex Weston) Duchesne ex Rozier (strawberry), 12 different compounds have been detected, and in *Vicia faba* L. (field bean), 11 different compounds have been detected (Table 2.3). In order to obtain a visual indication of the extent to which the compounds were detected in plant taxa, we created a heatmap for the PPP residues, with a Log₁₀ scale (Figure 2.2).

The map depicts the median concentrations of the PPP residues of both nectar and pollen in respective plant families (divided into the categories of cultivated and wild taxa), according to the intensity of a colour scale. The blank squares highlight where specific compounds were not evaluated for certain combinations (compound-family), emphasizing the amount of missing information. The majority of residues were generally below 10 ng/g, and the median concentrations of the compounds found in the cultivated plant families appeared to be higher (1.8 ng/g) than those in the wild plant families (0.04 ng/g). However, there is a lot of information missing, and in particular for the wild species, which are under-studied compared to the cultivated taxa.

Table 2.1. The chemical compounds evaluated for residues in pollen and nectar, their status in Europe and the United States, and the papers they were evaluated in.

Category	Substance group	Compound	Systemic	Current status		Citation ^a
				E.E ^b	U.S ^c	
Insecticides	Neonicotinoids	Acetamiprid	Yes	A ^d	A	8; 9; 17; 20
		Clothianidin	Yes	NA ^e	A	6; 8; 9; 13; 14; 15; 20; 23; 25
		Dinotefuran	Yes	NA	A	10
		Imidacloprid	Yes	RU ^f	- ^g	1; 4; 5; 6; 7; 8; 9; 10; 11; 12; 15; 16; 17; 19; 20; 21; 22
		Thiacloprid	Yes	A	-	6; 8; 9; 17; 20
		Thiamethoxam	Yes	NA	A	6; 8; 9; 10; 12; 13; 17; 20; 22
	Organophosphate	Chlorpyrifos	No	A	A	17
		Dimethoate	Yes	NA	A	3; 18; 24
		Phorate	Yes	NA	A	18
	Carbamate	Carbofuran	Yes	NA	A	2
		Oxamyl	Yes	A	A	10
	Pyrethroid	Cypermethrin	No	A	A	17
		Lambda-cyhalothrin	No	A	A	2; 17
	Natural product	Spinosad	Yes	A	A	2
Fungicides	Benzimidazole	Carbendazim	Yes	NA	A	8; 9; 17
	Carboxamide	Boscalid	Yes	A	A	8; 9; 17

	Chloronitrile	Chlorothalonil	No	NA	A	17
	Dicarboximide	Iprodione	Yes	NA	A	17
	Imidazole	Prochloraz	No	A	-	8; 9; 17
	Morpholine	Spiroxamine	Yes	A	-	8; 9; 17
	Oxathiin	Carboxin	Yes	A	A	8; 9
	Phenylamide	Metalaxyl	Yes	A	A	13
		Fluoxastrobin	Yes	A	A	8; 9; 17
	Strobilurin	Pyraclostrobin	Yes	A	A	8; 9; 17
		Trifloxystrobin	Yes	A	A	8; 9; 13; 17
	Thiophene	Silthiofam	Yes	A	-	8; 9
		Epoxiconazole	Yes	A	-	8; 9
		Flusilazole	Yes	NA	-	8; 9
	Triazole	Metconazole	Yes	A	A	8; 9
		Tebuconazole	Yes	A	A	8; 9; 17
		Triticonazole	Yes	A	A	8; 9
Metabolites		5-Hydroxy-imidacloprid	NA	-	-	19
		Imidacloprid olefin	NA	-	-	19
Synergist		Piperonyl butoxide	NA	-	-	8; 9

^a The complete citation is given in Supplementary material (see Appendix A Table A4), ^b European Union, ^c United States, ^d Active, ^e Not active, ^f Restricted use, ^g Information could not be acquired for these compounds.

Table 2.2. The chemical compounds evaluated for residues in pollen (P) and nectar (N), the number of families and species they were detected, their mean concentrations, the number of times they were detected in pollen and nectar, the mean range of limits of detection, and the number of studies that provided the limits of detection.

Compound	No. Families ^a	No. Species ^b	Range (ng/g)						Counts ^c		LOD range (ng/g)	
			Median residues (ng/g)		P		N		P	N	P	N
			P	N	Minimum	Maximum	Minimum	Maximum				
5-Hydroxy-imidacloprid	2	2	-	17.0	-	-	2.00	69.0	-	10	-	0.50
Acetamiprid	11	18	0.01	0.1	0.002	0.82	0.05	7.60	38	3	0.02-0.84	0.08-0.42
Boscalid	13	23	0.90	0.4	0.06	38.0	0.41	2.05	39	5	0.12-8.20	0.82-4.10
Carbendazim	11	17	2.50	1.3	0.01	204.0	1.25	1.25	37	1	0.08-24.00	2.40-12.00
Carbofuran	1	1	1400	-	1400	1400	-	-	2	-	20.00	-
Carboxin	8	13	0.06	-	0.01	0.06	-	-	33	-	0.12	-
Chlorothalonil	2	2	265.3	76.0	130.5	422	76.0	76.0	2	1	84.00-1521.00	152.00-760.00
Chlorpyrifos	2	2	100.5	-	38.00	163	-	-	2	-	21.00-377.00	-
Clothianidin	21	60	0.03	0.1	0.01	11.0	0.01	2992	214	64	0.12-0.72	0.17-1.00
Cypermethrin	1	1	58.5	-	58.5	58.50	-	-	1	-	24.00-430.00	-
Dimethoate	5	5	500	1595	500	500	100	22940	1	40	0.50	0.10
Dinotefuran	1	1	34.7	7.0	11.20	88.30	2.10	9.20	5	5	0.20	0.20
Epoxiconazole	8	13	0.42	-	0.10	31.0	-	-	33	-	0.84	-
Fluoxastrobin	10	15	0.01	0.01	0.001	0.15	0.01	0.01	35	1	0.01-0.27	0.03-0.13
Flusilazole	8	13	0.12	-	0.03	16.0	-	-	33	-	0.24	-
Imidacloprid	26	78	0.08	0.08	0.01	150	0.01	6588	240	111	0.05-10.00	0.05-3.60
Imidacloprid olefin	2	2	-	30.0	-	-	1.00	55.0	-	10	-	0.50
Iprodione	2	2	524	18.5	53.0	995.0	18.5	18.5	2	1	4.10-75.00	7.50-37.00
Lambda-cyhalothrin	2	2	30.0	-	30.0	57.5	-	-	3	-	11.00-202.00	-

Metalaxyl	1	1	3.10	-	3.10	3.10	-	-	1	-	0.50	-
Metconazole	8	13	0.15	-	0.04	19.00	-	-	33	-	0.30	-
Oxamyl	1	1	35.0	35.0	35.0	35.0	35.0	35.0	1	1	7.00	7.00
Phorate	2	2	-	53.5	-	-	7.00	100	-	2	-	-
Piperonyl butoxide	8	13	0.36	-	0.09	0.36	-	-	33	-	0.72	-
Prochloraz	13	20	0.18	0.30	0.04	46.00	0.30	0.30	40	2	0.33-6.00	0.60-3.00
Pyraclostrobin	11	17	0.12	0.60	0.03	19.60	0.60	0.60	35	2	0.24-12.00	1.20-6.00
Silthiofam	8	13	0.12	-	0.03	0.12	-	-	33	-	0.24	-
Spinosad	1	1	320	-	320	320	-	-	2	-	1.00	-
Spiroxamine	11	22	5.80	0.03	0.002	328	0.03	0.15	41	4	0.02-0.65	0.12-2.20
Tebuconazole	9	14	2.80	0.18	0.12	34.0	0.18	0.18	34	1	0.19-3.50	0.35-1.70
Thiacloprid	24	69	0.01	0.01	0.003	78.0	0.004	65.6	220	65	0.04-0.91	0.03-0.46
Thiamethoxam	21	68	0.06	0.05	0.01	95.2	0.01	11.0	230	74	0.10-1.80	0.10-2.00
Trifloxystrobin	10	15	0.12	1.20	0.03	104	1.20	1.20	35	1	0.24-4.80	0.48-2.40
Triticonazole	8	13	0.12	-	0.03	0.12	-	-	33	-	0.24	-
TOTAL									1491	404		

^a The number of families, ^b The number of species, ^c The number of records for reported residues.

Table 2.3. The plant taxa evaluated for PPP residues in pollen (P) and nectar (N), their status (cultivated or wild), the number of compounds that were detected on them, the mean concentration of residues, the number of studies that evaluated pollen and nectar, and the studies they were evaluated in.

Plant family	Plant species	Status		No. compounds			Median residues		Range (ng/g)				Counts ^a		Citation ^b
							(ng/g)								
		C ^c	W ^d	P	N	>LOD/LOQ ^e	P	N	P		N		P	N	
									Min	Max	Min	Max			
Amaryllidaceae	<i>Allium cepa</i> L.	1	-	-	1	1	-	1050	-	-	200	6900	-	14	24
	<i>Aethusa cynapium</i> L.	-	1	20	-	0	0.10	-	0.005	0.42	-	-	24	-	6; 9
Apiaceae	<i>Anthriscus sylvestris</i> (L.) Hoffm.	-	1	4	-	0	0.02	-	0.004	12.8	-	-	8	-	6
	<i>Heracleum sphondylium</i> L.	-	1	20	-	6	0.06	-	0.001	85.0	-	-	136	-	6; 9
	<i>Pimpinella saxifraga</i> L.	-	1	20	-	5	0.12	-	0.005	5.00	-	-	28	-	6; 9
Aquifoliaceae	<i>Ilex x attenuate</i>	1	-	-	3	3	-	10.00	-	-	1.00	2.76	-	15	19
Asparagaceae	<i>Muscari armeniacum</i> Leichtlin ex Baker	1	-	6	1	1	0.30	0.60	0.01	6.85	0.60	0.60	5	1	17
	<i>Ageratum houstonianum</i> Mill.	1	-	6	-	3	3.22	-	0.15	53.0	-	-	6	-	17
Asteraceae	<i>Arctium</i> sp.	-	1	4	4	0	0.01	0.02	0.005	0.04	0.005	0.03	8	4	6
	<i>Carduus</i> sp.	-	1	4	4	1	0.01	0.06	0.004	0.48	0.01	0.08	4	4	6
	<i>Centaurea nigra</i> L.	-	1	4	-	1	0.01	-	0.004	7.60	-	-	4	-	6
	<i>Centaurea scabiosa</i> L.	-	1	4	-	0	0.06	-	0.02	0.08	-	-	4	-	6
	<i>Cirsium arvense</i> (L.) Scop.	-	1	4	4	1	0.02	0.02	0.005	14.8	0.004	0.15	24	16	6

	<i>Cirsium vulgare</i> (Savi) Ten.	-	1	20	4	7	0.03	0.02	0.003	151	0.004	0.15	68	28	6; 9
	<i>Cosmos bipinnatus</i> Cav.	1	-	5	-	2	0.60	-	0.09	0.80	-	-	5	-	17
	<i>Coreopsis grandiflora</i> Sweet	1	-	4	1	0	0.80	0.60	0.06	38.0	0.60	0.60	3	1	17
	<i>Dahlia x hortensis</i> Guillaumin	1	-	1	-	1	0.30	-	0.30	0.30	-	-	1	-	17
	<i>Dahlia x hybrida</i> (1) ^f	1	-	1	-	0	0.04	-	0.04	0.04	-	-	1	-	17
	<i>Dahlia x hybrida</i> (2)	1	-	4	-	3	1.62	-	0.02	2.90	-	-	4	-	17
	<i>Helianthus annuus</i> L.	1	-	1	1	1	3.90	1.90	3.00	18.3	1.90	1.90	3	1	4; 16; 21
	<i>Hieracium</i> sp.	-	1	4	4	0	0.02	0.03	0.005	0.08	0.005	0.25	12	12	6
	<i>Jacobaea vulgaris</i> Gaertn.	-	1	20	-	5	0.06	-	0.003	17.13	-	-	84	-	6; 9
	<i>Matricaria discoidea</i> DC.	-	1	4	-	0	0.13	-	0.02	0.18	-	-	4	-	6
	<i>Matricaria chamomilla</i> L.	-	1	20	-	6	0.06	-	0.001	104	-	-	152	-	6; 9
	<i>Sonchus arvensis</i> L.	-	1	4	4	2	0.02	0.02	0.004	14.8	0.004	0.25	24	20	6
	<i>Sonchus oleraceus</i> L.	-	1	4	4	1	0.01	0.05	0.003	8.11	0.004	1.8	16	12	6
	Unidentified Asteraceae	-	1	4	-	1	0.01	-	0.005	11.7	-	-	4	-	6
Boraginaceae	<i>Myosotis arvensis</i> (L.) Hill	-	1	4	-	1	0.07	-	0.02	2.14	-	-	4	-	6
	<i>Brassica napus</i> L. (1)	1	-	-	3	3	-	2.60	-	-	0.10	4.40	-	5	20
Brassicaceae	<i>Brassica napus</i> L. (2)	1	-	20	5	14	0.18	0.50	0.005	328	0.05	65.6	226	13	6; 9; 11; 20; 25
	<i>Brassica rapa</i> (L.) L.	-	1	4	-	1	0.03	0.02	0.004	2.70	0.004	0.08	19	8	6
	<i>Cardamine pratensis</i> L.	-	1	4	-	0	0.04	-	0.01	0.06	-	-	4	-	6

	<i>Erysimum linifolium</i> (Pers.) J. Gay	1	-	2	2	0	66.27	38.60	2.05	130.5	1.20	76.0	2	2	17
Campanulaceae	<i>Campanula portenschlagiana</i> Schult.	1	-	3	-	1	0.49	-	0.10	0.90	-	-	3	-	17
Caprifoliaceae	<i>Scabiosa columbaria</i> L.	1	-	-	2	0	-	0.28	-	-	0.06	0.50	-	2	17
	<i>Silene dioica</i> (L.) Clairv.	-	1	4	4	0	0.03	0.06	0.01	0.09	0.01	0.08	4	4	6
Caryophyllaceae	<i>Silene latifolia</i> Poir.	-	1	20	4	8	0.12	0.08	0.005	138	0.01	0.15	88	8	6; 9
	<i>Silene vulgaris</i> (Moench) Garcke	-	1	4	4	1	0.01	0.06	0.004	12.8	0.04	0.25	4	4	6
Clethraceae	<i>Clethra alnifolia</i> L.	1	-	-	3	3	-	40.0	-	-	12.0	515	-	15	19
	<i>Calystegia sepium</i> (L.) R. Br.	-	1	4	4	0	0.02	0.02	0.01	0.04	0.005	0.03	8	4	6
Convolvulaceae	<i>Calystegia silvatica</i> (Kit.) Griseb.	-	1	4	4	1	0.03	0.01	0.01	6.14	0.004	0.06	12	4	6
	<i>Convolvulus arvensis</i> L.	-	1	4	4	1	0.02	0.02	0.005	17.1	0.004	0.03	16	8	6
	<i>Bryonia dioica</i> Jacq.	-	1	4	-	0	0.06	-	0.02	0.08	-	-	4	-	6
	<i>Cucurbita pepo</i> L. (1)	1	-	4	4	3	25.2	6.10	0.1	95.2	0.10	11.20	19	19	10
Cucurbitaceae	<i>Cucurbita pepo</i> L. (2)	1	-	2	2	2	13.0	10.5	12.0	14.0	10.00	11.00	4	4	22
	<i>Cucurbita pepo</i> L. (3)	1	-	2	2	2	13.0	10.5	12.0	14.0	10.00	11.00	4	4	22
	<i>Cucurbita pepo</i> L. (4)	1	-	2	2	2	13.0	10.5	12.0	14.0	10.00	11.00	4	4	22
Dipsacaceae	<i>Knautia arvensis</i> (L.) DC.	-	1	4	4	0	0.06	0.02	0.02	0.08	0.005	0.03	4	4	6
Ericaceae	<i>Erica carnea</i> L.	1	-	10	-	6	26.5	-	0.49	422	-	-	10	-	17
Fabaceae	<i>Medicago sativa</i> L.	1	-	1	1	1	500	1875	500	500	100.00	22940	1	23	3
	<i>Trifolium pratense</i> L.	-	1	4	-	2	0.51	-	0.004	12.6	-	-	4	-	6

	<i>Trifolium repens</i> L.	1	1	4	4	4	0.03	6.20	0.01	0.08	0.004	6588	12	17	6; 14; 15
	<i>Vicia faba</i> L.	1	-	20	2	11	0.28	10503.5	0.01	4.20	7.00	21000	20	2	8; 18
	<i>Vicia sativa</i> L.	-	1	4	-	2	0.51	-	0.004	12.6	-	-	4	-	6
Hypericaceae	<i>Hypericum hirsutum</i> L.	-	1	4	-	1	0.07	-	0.02	7.47	-	-	4	-	6
	<i>Crocus vernus</i> (L.) Hill	1	-	2	2	0	0.97	0.33	0.39	155	0.30	0.36	2	2	17
Iridaceae	<i>Iris tingitana</i> Boiss. & Reut. ×	1	-	4	4	2	0.10	0.14	0.01	7.20	0.01	1.25	4	4	17
	<i>Iris xiphium</i> L.														
	<i>Ballota nigra</i> L.	-	1	4	-	1	0.01	-	0.004	17.1	-	-	12	-	6
	<i>Glechoma hederacea</i> L.	-	1	4	4	2	0.03	0.02	0.004	12.6	0.005	0.03	8	4	6
	<i>Lamium album</i> L.	-	1	4	4	2	0.51	0.02	0.004	12.6	0.005	0.03	4	4	6
	<i>Lamium purpureum</i> L.	-	1	4	3	2	0.06	0.02	0.004	12.6	0.005	0.03	11	3	6
	<i>Lavandula stoechas</i> L.	1	-	1	2	1	19.6	0.35	19.6	19.6	0.03	0.41	1	2	17
Lamiaceae	<i>Nepeta cataria</i> L. (1)	1	-	-	4	0	-	0.23	-	-	0.03	1.20	-	4	17
	<i>Nepeta cataria</i> L. (2)	1	-	-	3	0	-	0.41	-	-	0.03	1.20	-	3	17
	<i>Salvia longispicata</i> M. Martens														
	& Galeotti x <i>Salvia farinacea</i>	1	-	-	2	0	-	0.10	-	-	0.07	0.14	-	2	17
	Benth.														
	<i>Stachys sylvatica</i> L.	-	1	4	-	1	0.01	-	0.004	12.8	-	-	4	-	6
	<i>Gossypium</i> sp.	1	-	2	2	2	7.25	0.40	2.9	33.1	0.30	0.90	4	4	12
Malvaceae	<i>Malva moschata</i> L.	-	1	4	4	0	0.07	0.02	0.02	0.18	0.005	0.03	4	4	6
	<i>Malva sylvestris</i> L.	-	1	4	4	1	0.03	0.02	0.01	6.14	0.004	0.06	12	8	6

Oleaceae	<i>Ligustrum vulgare</i> L.	-	1	4	-	0	0.03	-	0.01	0.04	-	-	8	-	6
Onagraceae	<i>Epilobium hirsutum</i> L.	-	1	4	4	1	0.02	0.04	0.004	7.60	0.005	0.08	24	12	6
	<i>Fuchsia</i> sp.	1	-	-	2	2	-	1495	-	-	100	2890	-	2	18
Papaveraceae	<i>Fumaria officinalis</i> L.	-	1	4	-	1	0.03	-	0.004	0.48	-	-	20	-	6
	<i>Papaver rhoeas</i> L.	-	1	20	-	4	0.04	-	0.003	64.1	-	-	56	-	6; 9
	<i>Digitalis purpurea</i> L.	1	-	2	2	0	1.23	0.44	0.07	2.40	0.03	0.85	2	2	17
Plantaginaceae	<i>Microrrhinum minus</i> (L.) Fourr.	-	1	4	4	0	0.02	0.02	0.005	0.08	0.005	0.03	8	4	6
	<i>Veronica persica</i> Poir.	-	1	4	-	1	0.01	-	0.003	8.11	-	-	12	-	6
	<i>Veronica spicata</i> L.	1	-	6	6	3	2.87	1.62	0.10	995	0.15	18.5	6	6	17
Poaceae	<i>Zea mays</i> L.	1	-	8	-	8	6.60	-	1.7	1400	-	-	15	-	1; 2; 4; 5; 13; 25
Ranunculaceae	<i>Clematis vitalba</i> L.	-	1	4	4	2	0.06	0.01	0.005	10.3	0.004	0.02	8	4	6
	<i>Ranunculus repens</i> L.	-	1	20	-	3	0.12	-	0.01	8.50	-	-	24	-	9
	<i>Agrimonia eupatoria</i> L.	-	1	4	-	1	0.01	-	0.004	7.60	-	-	4	-	6
	<i>Fragaria x ananassa</i> (Duchesne ex Weston) Duchesne ex Rozier	1	-	21	-	12	0.46	-	0.01	58.5	-	-	24	-	8; 17
Rosaceae	<i>Rosa arvensis</i> Huds.	-	1	4	4	2	0.53	0.02	0.005	10.6	0.005	0.03	4	4	6
	<i>Rosa canina</i> L.	-	1	4	4	1	0.04	0.06	0.04	0.32	0.01	0.08	4	4	6
	<i>Rubus fruticosus</i> agg.	-	1	5	4	3	0.02	0.04	0.004	11.7	0.01	0.25	38	12	6; 8
	<i>Rubus</i> sp.	1	-	20	-	10	0.91	-	0.005	23.0	-	-	20	-	8
Rutaceae	<i>Citrus × sinensis</i> (1)	1	-	-	1	1	-	15.4	-	-	1.79	21.2	-	8	7

	<i>Citrus × sinensis</i> (2)	1	-	-	1	1	-	21.2	-	-	0.80	21.2	-	6	7
	<i>Citrus reticulata</i> Blanco	1	-	-	1	1	-	6.82	-	-	6.82	6.82	-	1	7
Solanaceae	<i>Datura stramonium</i> L.	-	1	4	-	0	0.01	-	0.005	0.02	-	-	4	-	6
	<i>Solanum dulcamara</i> L.	-	1	4	-	1	0.01	-	0.004	17.1	-	-	8	-	6
Tropaeolaceae	<i>Tropaeolum</i> sp.	1	-	-	1	1	-	741	-	-	741	741	-	1	18
Violaceae	<i>Viola arvensis</i> Murray	-	1	4	4	2	0.03	0.02	0.005	3.78	0.004	0.03	20	12	6
TOTAL		41	54										1491	404	

^a The number of records for reported residues, ^b The complete citation is given in Supplementary material (see Appendix A Table A4), ^c Cultivated, ^d Wild, ^e Values of residues above the limits of detection/quantification, ^f The numbers refer to different varieties or cultivars (e.g. winter or spring), more information in Supplementary material (see Appendix A Table A1).

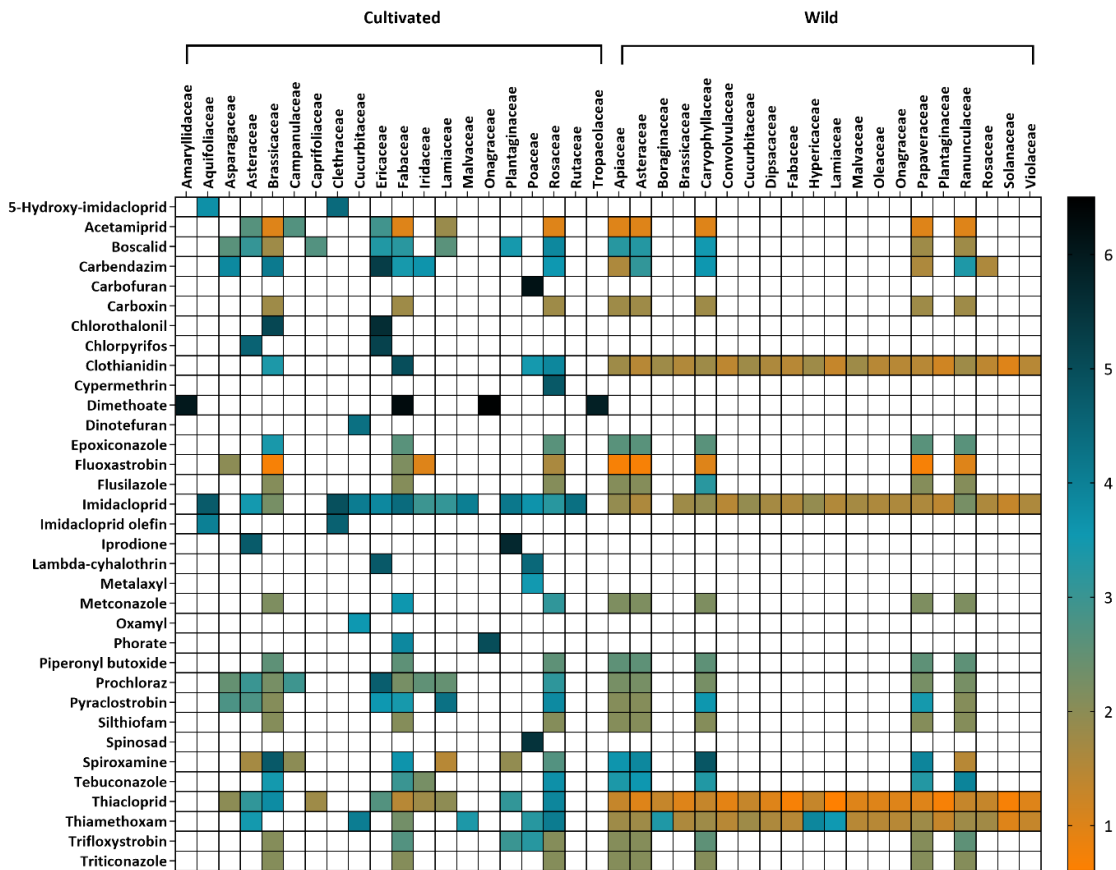


Figure 2.2. A heat map on a Log_{10} scale (colour scale on the right), showing the median concentrations of the PPP residues (left y axis) for both nectar and pollen in respective plant families (top x axis). The plant families are separated in two categories: cultivated and wild taxa. The cool colours represent high residue concentrations, while the warmer colours represent lower concentrations. The blank squares highlight the compounds that were not evaluated for certain families, emphasizing the amount of missing information. In order to avoid negative Log_{10} values, and for the purposes of this figure only, we converted the values of the residue concentrations in pg/g instead of ng/g .

2.4.3. Extent of PPP residues in pollen and nectar

The majority of studies evaluated pollen as a matrix for detecting PPP residues (Figure 2.1a), both overall and when evaluating across the various plant taxa, with 1,491 reports for PPP residues present in pollen (compared with 404 reports for residues present in nectar) (Table 2.3). For both pollen and nectar, the majority of residue values were reported as < LOD/LOQ (78% for pollen and 64% for nectar). Pollen was the matrix in which the majority of the different PPPs were detected ($n_{\text{pollen}} = 31$ and $n_{\text{nectar}} = 21$). However, the individual studies differed in their application rates and types, and rarely if ever included control treatments. This made it difficult to compare between studies and hence, we were restricted to using only the studies with paired residue concentrations for pollen and nectar. Therefore, out of all the studies we were limited to seven for statistical analysis, and out of all the residue reports, we could only use the 206 reports that referred to both pollen and nectar residues. In these studies, research was performed on five compounds that belonged to the category of insecticides (clothianidin, dinotefuran, imidacloprid, thiacloprid and thiamethoxam) and on 13 plant families. Our model results indicated a positive correlation between the natural log of residues in nectar and pollen for these five insecticides ($\beta = 0.593 \pm 0.084$, $p < 0.001$ in cultivated plants). The slope of this relationship was less steep in wild than cultivated species, ($\beta = -0.469 \pm 0.092$, $p < 0.001$). However, the estimate of this slope for wild species may be influenced by the high proportion of measurements of both pollen and nectar that were below the limits of detection in wild plant species. For this dataset comprised of paired data, concentrations of residues were generally lower in wild than cultivated plants (Figure 2.3). Concentrations observed in nectar also differed significantly between compounds (see Appendix A Table A5). This difference was primarily driven by the lower concentrations of the insecticide thiacloprid, but this may be due to application rate or other confounding factors rather than the properties of the compound itself. The amount of variation in log concentrations in nectar explained in the full model was 88%, with 78% of this variation being explained by the fixed factors (pollen, compound type, and status (cultivated/wild)). Differences between studies and plant families, explained 5.4% and 4.5% of the model variance respectively.

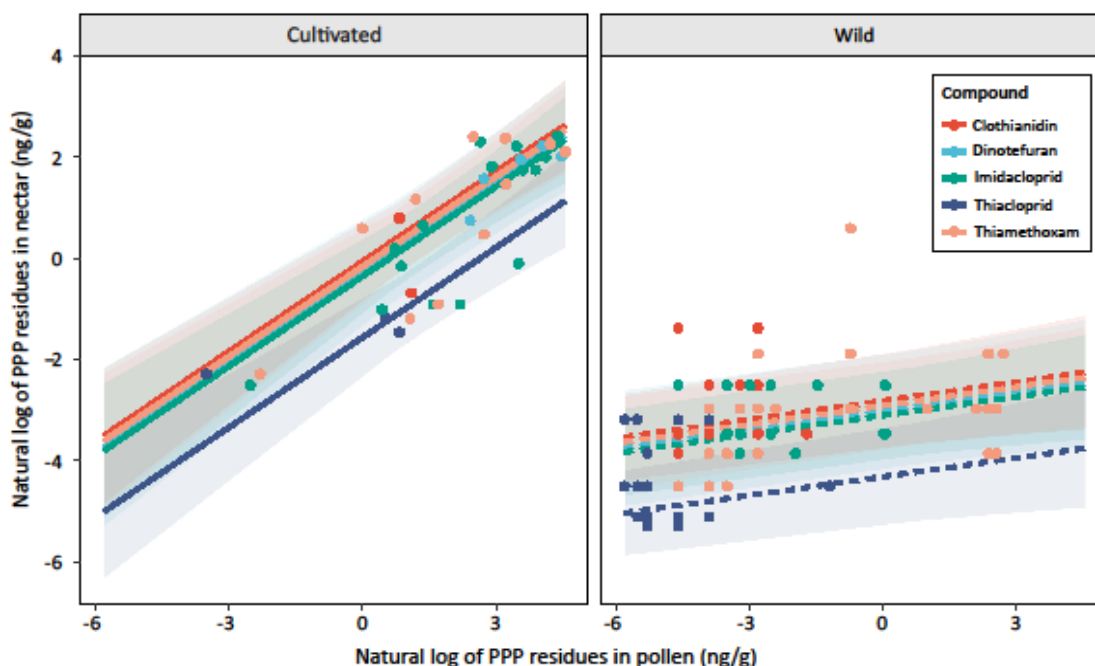


Figure 2.3. PPP residues in nectar and pollen for cultivated and wild plant species. Points show data from studies where both nectar and pollen were measured concurrently. Colours indicate PPP compounds investigated. Fitted lines show the relationship between pollen and nectar residues from the Generalised Linear Mixed Model. Shaded areas indicate the 95% confidence intervals on fitted lines. Dashed lines in the wild species plot should be interpreted with caution as the majority of residues were below the limits of detection for wild species (see main text).

2.4.4. PPP residues and toxicity to bees

LD₅₀s from toxicity assay data were available for honey bees (*Apis* spp.), bumble bees (*Bombus* spp.) and six species of solitary bees (*Osmia* spp., *Megachile rotundata*, *Scaptotrigona postica*, *Nannotrigona perilampoides*, *Trigona* sp. and *Nomia melanderi*) (University of Hertfordshire, 2019). From this initial assessment, a forager honey bee may daily be exposed to 50-100% (84%) of its LD₅₀ for spinosad through both oral and contact exposure to pollen and greater than its LD₅₀ for dimethoate, imidacloprid and clothianidin through both oral and contact exposure to nectar (Table 2.4.1). A bumble bee could daily be exposed to 50-100% (70%) of its LD₅₀ for thiamethoxam, and greater than its LD₅₀ for the compounds imidacloprid and dimethoate through nectar consumption and come in contact with greater than its LD₅₀s for the compounds clothianidin, imidacloprid and dimethoate. Our results show that the residues of the compound dimethoate found in pollen can cause contact toxicity to the solitary bee species *Nomia melanderi*, while having very low % LD₅₀ values for other bee species. The same applies for thiacloprid residues found in nectar and the solitary bee species *Nannotrigona perilampoides* (Table 2.4.2). A nectar foraging honey bee over a single foraging bout is likely to be exposed to

concentrations above its LD_{50s} for the compounds dimethoate, imidacloprid, clothianidin, thiamethoxam, dinotefuran, phorate and oxamyl (see Appendix A Table A3).

2.5. Discussion

In the present study, we performed a literature review of the PPP residues detected in nectar and pollen from plants. Most publications originate from North America (mainly United States) and Europe (mainly United Kingdom), while there is complete absence of studies from South America, most of Asia, Africa and Australia, creating a restricted view of the field realistic exposure to PPPs and leading to big knowledge gaps. Hence, there is an urgent need for reporting the field realistic residues from countries of the southern hemisphere and Asia since their particular climatic conditions along with the different agricultural practices and bee species may produce different conclusions from the ones that have been drawn for the Northern hemisphere (Benuszek et al., 2017).

The first reports of PPP residues in nectar date back in the '60s (Lord et al., 1968), and until the '80s they were mainly focused on the organophosphate compound dimethoate. A significant amount of time passed (20 years) without any additional reports. After the introduction of the neonicotinoid compound imidacloprid to the market (Bonmatin et al., 2003), and the intense concerns associating its widespread use with honey bee deaths (Bonmatin et al., 2005), imidacloprid residues were reported in nectar and pollen for the first time in 2001. However, the majority of studies of residues in nectar and pollen were published the years 2012 and 2015, emphasizing the recent concerns regarding the environmental fate and the effects of the chemicals to pollinators (Benuszek et al., 2017). Fungicides were evaluated in nectar and pollen for the first time in 2012 but gained more attention after 2015. This is because until 2015, the acute toxicity of fungicides to adult bees was assumed to generally be low (Johnson, 2015), although it was suspected that they may act synergistically with systemic insecticides, increasing their toxicity by as much as a factor of 1000 (Iwasa et al., 2004; Schmuck et al., 2003). Despite the fact that herbicides have been detected when analysing mixed matrices containing nectar from flowers, nectar stored in the comb, and honey or pollen from pollen traps (Pohorecka et al., 2012; Prado et al., 2019), the presence of herbicides in raw pollen or nectar collected from plants has neither been verified nor evaluated, highlighting that future research needs to address this issue. Insecticides require higher tier risk assessments since they are more toxic to insects (Kyriakopoulou et al., 2017; Sánchez-Bayo and Goka, 2014). However, evidence is emerging that compounds belonging to the category of herbicides were found to implicate sublethal effects on pollinators (Bohnenblust et al., 2016; Cousin et al., 2013, Dai et al., 2018;

Faita et al., 2018; Farina et al., 2019; Mengoni Goñalons and Farina, 2018; Helmer et al., 2014; Jumarie et al., 2017; Motta et al., 2018; Seide et al., 2018; Vázquez et al., 2018), thus they should also be evaluated for field realistic residues.

We found 31 fungicides and insecticides identified in pollen and nectar collected directly from plants. Even though more fungicide compounds were examined for PPP residues overall, insecticides were more commonly evaluated, particularly, neonicotinoids. Exposure to neonicotinoids has been cited as an exceptional cause for concern for pollinating insects, because they are widely used systemic agrochemicals that have been shown to contaminate pollen and nectar of crop plants and nearby wildflowers (Bonmatin et al., 2015; Botías et al., 2015, 2016; Chen et al., 2013; Choudhary and Sharma, 2008; Cowles and Eitzer, 2017; Cutler and Scott-Dupree, 2014; Fairbrother et al., 2014; Goulson et al., 2015; Hoffmann and Castle, 2012; Pilling et al., 2013; Tosi et al., 2018; Xu et al., 2016). However, as identified here, none of the compounds with the highest median levels of residues in pollen and nectar (carbofuran, iprodione, dimethoate, spinosad, chlorothalonil, phorate and oxamyl) belongs to the neonicotinoid category. Moreover, for a risk assessment it is very important to collect extended data on various chemical compounds (Benuszek et al., 2017), rather than focusing on just a few. The total number of publications concerning PPP residues in nectar and pollen directly collected from plants (n = 25 studies) and the respective number of PPPs evaluated remains low, given that approximately 1000 active substances are globally available (Lewis et al., 2016). However, since it is not possible to test all potential combinations, the mixtures most likely to occur in the agricultural areas of a country should be considered (Sgolastra et al., 2020; Zhu et al., 2017b).

Research to date has focused predominantly on parent active substances alone, and there is evidence for known concentrations of only two metabolites of the active substance imidacloprid (5-Hydroxy-imidacloprid and imidacloprid olefin) and one synergist (piperonyl butoxide), confirming that very few studies evaluate the presence of the main compound degradation products and the ingredients of the commercial formulations (Benuszek et al., 2017). Our review highlights that as the majority of PPP were applied as commercial formulations, co-formulants should therefore be expected to be present, with the most frequent co-formulants found to be three fungicides, two insecticides and one preservative with fungicidal properties. This creates more concerns, since it has been found that co-formulants may have a negative effect on honey bees (Mullin, 2015; Mullin et al., 2015), while it is known that some metabolites of active substances may have higher toxicity than the initial compound (Nauen et al., 2001).

Contrary to the limited number of compounds evaluated, a wide range of plant taxa, including both cultivated and wild plants, were studied for PPP residues in nectar and pollen. As illustrated in the heatmap (Figure 2.2), it is evident that the concentrations of the compounds found in cultivated plant taxa are higher from those found in the wild. However, there is a lot of information missing as wild species are significantly understudied compared to cultivated. In spite of the fact that, in total, more wild taxa than cultivated have been studied, the cultivated taxa were more often evaluated, while the information about residues found in wild species derives from only two studies (Botías et al., 2015; David et al., 2015, 2016). According to recent research, wild plants growing in close proximity to agricultural land can be a source of higher and more prolonged PPP exposure than the crops (Botías et al., 2015; Wood et al., 2019). Thus, for future residue studies, it is suggested to investigate both cultivated and wild species, preferably in the same study.

In their discussion of determination of PPP residue concentrations in bee relevant matrices in field experiments, it was acknowledged by US regulators that a primary limitation was that both spatial and temporal resolution of the PPP residue profiles in these matrices was low (Heimbach and Schmuck, 2017). This is also highlighted in the relevant limited research published. For the chemical analyses of pollen and nectar a minimum of approximately 100 mg (pollen) (Botías et al., 2015) and 10 μ L (nectar) (Martel et al., 2013) is required. However, even when these amounts are collected, this may not be sufficient to detect or quantify PPP residues: in the full dataset analysed in our study, the majority of reports for both matrices were reported as below the values of limits of detection or quantification (LOD or LOQ). It is clear therefore that for pollen and nectar analysis in field experiments, significant limitations exist in terms of both sampling requirements and analytical instrumentation capabilities. The low amounts of the acquired matrices are also the reason why a multi residue analysis cannot be easily performed, and the maximum number of PPPs analysed was 20. Moreover, the available studies differed considerably in design, sampling timing, sampling methodology and application scenarios, or lacked data types of active substances, meaning residue data were difficult to compare (Gierer et al., 2019). Hence, in order to evaluate if there is a relationship between the two matrices, we chose to perform an analysis with a selected amount of paired data and only for the compounds for which we had enough information. Strong positive correlation was observed between pollen and nectar insecticide residues in cultivated plant species, and this is in accordance with previous findings (Kyriakopoulou et al., 2017). Taking into account the fact that the volume of pollen needed for analysis can be collected more easily than that of nectar, and the fact that there is a correlation between those two matrices - at least for the group of neonicotinoid

insecticides, we suggest that pollen could be used as surrogate matrix for the analysis of neonicotinoid residues in cultivated taxa. However, the correlations for wild species were weaker. This may have been influenced by the high proportion of measurements of both pollen and nectar, which were below the limits of detection in wild plant species (82% for pollen and 99% for nectar). For cultivated species with reliable estimates (i.e., above LOD/LOQ values are available), the vast majority (> 90%) had higher residues in pollen than in nectar, potentially due to its physical characteristics (i.e., highly sculptured cavities filled with a lipophilic pollen coat) (Mullin et al., 2010). In wild species, the levels of both were mostly below LOD/LOQ, but in pollen more frequently above LOD/LOQ than in nectar. Our analysis suggests that residues in wild plants were generally lower than in cultivated plants (especially in nectar). However, the current data does not permit further assumptions about the relationship between pollen and nectar of wild plants. It should be noted that it is typically much more difficult to collect sufficient pollen and nectar of wild plants for analysis. Pollen resources have been determined to be between 0.0001 μL and $15.9 \pm 2 \mu\text{L}$ per floral unit for pollinator friendly seed mixes (Hicks et al., 2016). Thus, in order to detect and quantify the PPP residues in pollen and nectar, relatively large amounts of the two matrices need to be collected from several plants (~ 1000 flowers). Given that wild species may not occur at high abundance in agricultural landscapes, reaching the threshold amount of matrix for analysis is challenging, potentially limiting the extent of our knowledge of residues in wild plants. Differences between plant families in terms of residue concentrations explained a relatively low amount of variance in the model (4.5% out of a total of 88% explained by the full model). However, more research should be carried out for other PPP categories (fungicides and herbicides), in order to examine whether the same relationship is applicable. The fact that 'Study' explained a lot of variation in residues measured in nectar emphasises the need for the standardization of protocols for experimental and analytic methods across studies on a global scale. Furthermore, the factor 'Status' was similarly important, and suggests that important differences may exist between wild and cultivated taxa, indicating that studies should examine the same compound on both cultivated (e.g., *B. napus*) and wild (e.g., *Rubus fruticosus* agg.) plant taxa.

By relating the maximum residue values of the PPPs found in nectar and pollen with the contact and oral LD_{50s} from toxicity assays for bees using the Estimated % LD_{50s}, we found that bees could be exposed to a high percentage of a toxic dose of certain compounds. For example, a nectar foraging honey bee over a single foraging bout is likely to be exposed to values that exceed the honey bee LD_{50s} for the compounds dimethoate, imidacloprid, clothianidin, thiamethoxam, dinotefuran, phorate and oxamyl (see Appendix A Table A3). In the present

review, we indicate that a mean number of two compounds was detected in each plant species, while a single cultivated (*Brassica napus*) or wild (*Silene latifolia*) plant has been found to be contaminated with up to 14 and 8 different compounds respectively. This implies that a nectar foraging honey bee could be exposed to a poisonous cocktail of PPPs if they forage on multiple plant taxa during a single foraging bout. Research has shown that short-term (acute) exposure to insecticides during one foraging bout can significantly impair learning and memory in bees (Siviter et al., 2018). Moreover, this could also mean that a generalist bee (a bee that visits a wide range of plant species) might be exposed to a higher number of PPPs during her lifespan (chronic exposure). This may also occur through repeated foraging on a large PPP-treated food source that flowers over a prolonged period, such as oilseed rape, and may be extended by the presence of PPPs within honey and pollen stores (Mitchell et al., 2017). According to our calculations, all the three bee types (honey bees, bumble bees and solitary bees) are likely to be exposed to values above the respective bee type LD_{50s} for several compounds (Tables 2.4.1 and 2.4.2). Exposure to combinations of various types of PPPs, either during a single foraging bout, or throughout their life cycle, can have a variety of negative impacts on their health (Gill et al., 2012; Lentola et al., 2017; Sanchez-Bayo et al., 2016). It has also been reported that many compounds become more toxic with repeated exposure over time, so that even low concentrations can eventually result in death (Rondeau et al., 2014; Sánchez-Bayo and Goka, 2014; Suchail et al., 2001). However, levels of contaminant mixtures can vary widely depending on the location and the type of plants (cultivated or wild), as well as the diet breadth and foraging area of the bee (Botías et al., 2015), and unfortunately there is limited data regarding the exact amount of flowers of a certain plant species that a bee might visit on a single bout, in order to be able to make assumptions on the exact level of exposure.

Our results indicate that residues of compounds found in pollen (such as dimethoate) or nectar (such as thiacloprid), that may have low Estimated % LD₅₀ values for honey bees and bumble bees, can have high Estimated % LD₅₀ values for solitary bee species (e.g., *Nomia melanderi* and *Nannotrigona perilampoides*). This highlights that we need to expand toxicity assays to more bee species and stresses that using honey bees as a surrogate for PPP risk assessment for all bee species is insufficient (Bireley et al. 2019; Boyle et al., 2019; Cham et al., 2019; Hinarejos et al., 2019; Sgolastra et al., 2019, 2020). It should also be noted that the compounds detected in high concentration levels in pollen and/or nectar (carbofuran, iprodione, dimethoate, spinosad, and chlorothalonil, phorate and oxamyl), did not necessarily have high Estimated % LD₅₀ values.

Table 2.4.1. The percentages of the Estimated % LD₅₀ based on the maximum residues of the PPP detected in pollen (P) and nectar (N) and the respective oral (O) and contact exposure (C) LD_{50s} from toxicity assays for honey bees and bumble bees.

Compound	Estimated % LD ₅₀ ^a							
	Honey bees				Bumble bees			
	<i>Apis</i> spp.				<i>Bombus</i> spp.			
	C ^b		O ^b		C		O	
	P	N	P	N	P	N	P	N
Acetamiprid	<50	<50	<50	<50	<50	<50	<50	<50
Boscalid	<50	<50	<50	<50	-	-	-	-
Carbendazim	<50	<50	<50	<50	-	-	-	-
Carbofuran	<50	-	<50	-	-	-	-	-
Carboxin	<50	-	<50	-	-	-	-	-
Chlorothalonil	<50	<50	<50	<50	<50	<50	<50	<50
Chlorpyrifos	<50	-	<50	-	<50	-	<50	-
Clothianidin	<50	>100 ^c	<50	>100	<50	>100	-	-
Cypermethrin	<50	-	<50	-	<50	-	<50	-
Dimethoate	<50	>100	<50	>100	<50	>100	<50	>100
Dinotefuran	<50	<50	-	-	-	-	-	-
Epoxiconazole	<50	-	<50	-	<50	-	<50	-
Fluoxastrobin	<50	<50	<50	<50	<50	<50	<50	-
Flusilazole	<50	-	<50	-	-	-	-	-
Imidacloprid	<50	>100	<50	>100	<50	>100	<50	>100
Iprodione	<50	<50	<50	<50	-	-	-	-
Lambda-cyhalothrin	<50	-	<50	-	<50	-	<50	-
Metalaxyl	<50	-	<50	-	-	-	-	-
Metconazole	<50	-	<50	-	<50	-	<50	-
Oxamyl	<50	<50	<50	<50	<50	<50	<50	<50
Phorate	-	<50	-	<50	-	<50	-	-
Piperonyl butoxide	<50	-	-	-	-	-	-	-
Prochloraz	<50	<50	<50	<50	-	-	-	-
Pyraclostrobin	<50	<50	<50	<50	<50	<50	<50	<50
Silthiofam	<50	-	<50	-	-	-	-	-

Spinosad	50-100^d	-	50-100	-	-	-	-	-
Spiroxamine	<50	<50	<50	<50	-	-	-	-
Tebuconazole	<50	<50	<50	<50	-	-	-	-
Thiacloprid	<50	<50	<50	<50	<50	<50	-	-
Thiamethoxam	<50	<50	<50	50-100	<50	<50	50-100	50-100
Trifloxystrobin	<50	<50	<50	<50	-	-	-	-
Triticonazole	<50	-	<50	-	-	-	-	-

^a The Estimated % LD₅₀ was calculated using the maximum PPP concentrations and the respective oral and contact exposure LD_{50s} from toxicity assays of the various bee species. ^b The information about the LD_{50s} was retrieved from the Pesticide Properties DataBase, developed by the Agriculture & Environment Research Unit (AERU) at the University of Hertfordshire, ^c The values highlighted in dark blue shading and bold white font (>100) indicate high percentages exceeding the Estimated % LD₅₀, ^d The values highlighted in light blue shading and bold black font (50-100) indicate values exceeding the 50% LD₅₀, but below the 100% LD₅₀.

In fact, it was mainly the neonicotinoid compounds that had high Estimated % LD₅₀ values, despite their low concentrations detected in pollen and nectar. This implies that compounds with similar properties to those of neonicotinoids (designed to have high efficacy, long persistence, high systemicity, high mobility, and application versatility) (Sgolastra et al., 2020), are likely to be more toxic to bees than others. The compounds that showed high Estimated % LD_{50s} were all insecticides, systemic and still approved for use in the United States, albeit only two of them are still approved for use on a European level (oxamyl and spinosad). Since there is not enough available data on other, non-neonicotinoid insecticide groups (e.g., pyrethroids, phosphorothioates, sulfoximines etc.) (Siviter et al., 2018), future studies should start investigating more insecticides both for their effects on bee species and for their residues in nectar and pollen.

Table 2.4.2. The percentages of the Estimated % LD₅₀ based on the maximum residues of the PPP detected in pollen (P) and nectar (N) and the respective oral (O) and contact exposure (C) LD₅₀s from toxicity assays for solitary bees.

Compound	Estimated % LD ₅₀ ^a															
	Solitary bees															
	<i>Osmia</i> spp.				<i>Megachile rotundata</i>				<i>Scaptotrigona postica</i>		<i>Nannotrigona perilampoides</i>		<i>Trigona</i> sp.		<i>Nomia melanderi</i>	
	C		O		C		O		O		C		C		C	
	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N
Acetamiprid	<50	<50	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Boscalid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbendazim	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbofuran	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carboxin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlorothalonil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlorpyrifos	<50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clothianidin	<50	>100	<50	<50	-	-	<50	<50	-	-	-	-	-	-	-	-
Cypermethrin	-	-	-	-	-	-	-	-	-	-	-	-	<50	-	-	-
Dimethoate	<50	>100	<50	>100	>100	>100	-	-	-	-	-	-	-	-	>100	>100
Dinotefuran	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epoxiconazole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fluoxastrobin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Flusilazole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Imidacloprid	<50	>100	-	-	<50	>100	-	-	<50	<50	-	-	-	-	-	-
Iprodione	<50	<50	<50	<50	-	-	-	-	-	-	-	-	-	-	-	-

Lambda-cyhalothrin	<50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Metalaxyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Metconazole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxamyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phorate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Piperonyl butoxide	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Prochloraz	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pyraclostrobin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Silthiofam	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spinosad	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spiroxamine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tebuconazole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thiacloprid	<50	<50	-	-	-	-	-	-	-	-	<50	>100	-	-	-	-	-
Thiamethoxam	-	-	-	-	-	-	-	-	-	-	<50	<50	<50	<50	-	-	-
Trifloxystrobin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Triticonazole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

^a The Estimated % LD₅₀ was calculated using the maximum PPP concentrations and the respective oral and contact exposure LD_{50s} from toxicity assays of the various bee species. ^b The information about the LD_{50s} was retrieved from the Pesticide Properties DataBase, developed by the Agriculture & Environment Research Unit (AERU) at the University of Hertfordshire, ^c The values highlighted in dark blue shading and bold white font (>100) indicate high percentages exceeding the Estimated % LD₅₀, ^d The values highlighted in light blue shading and bold black font (50-100) indicate values exceeding the 50% LD₅₀, but below the 100% LD₅₀.

2.6. Conclusions and future research

Based on our findings, major knowledge gaps in this field have been identified: 1. There is paucity of information from extensive geographical areas of the world and thus we have incomplete information on how the same compounds behave in different climates and landscapes. 2. There is an urgent need to expand the range of compounds analyzed in these studies. Herbicide and fungicide residues in pollen and nectar should be evaluated, alongside insecticides. Moreover, co-formulants used in PPPs along with the main active substances, and certain metabolites that derive from the degradation of the active compounds of those products, should be evaluated too. 3. As important differences may exist between cultivated and wild taxa, we suggest that at least one model wild plant species growing in the margins of the target crop should be evaluated in the same study. From our findings, *Rubus fruticosus* agg. (wild species), and *Trifolium repens* (both) are the plant taxa most widely evaluated, hence they could act as model taxa. 4. PPP residues in pollen and nectar are affected by plant taxon, application method, physicochemical properties of the active substance or the formulation, and environmental conditions. However, very few of the studies reported all this information, which precludes further evaluation of the fate of the compounds in pollen and nectar. We strongly advise that future research include detailed information of all these factors, and especially the study design, sampling points and their spatial and temporal distribution. 5. In cultivated plant species, the residues found in pollen were positively correlated with those found in nectar, hence, risk assessments could be based on the concentrations found in pollen. Overall, given that maximum concentrations of several compounds detected in nectar and pollen were estimated to exceed the LD_{50s} for several species of bee, nectar and pollen residues represent an ongoing risk to flower-feeding insects and need to be better studied before being properly integrated into risk assessment.

Chapter 3

Pesticide mixtures detected in crop and non-target wild plant pollen and nectar



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3. Pesticide mixtures detected in crop and non-target wild plant pollen and nectar

3.1. Abstract

Cultivation of mass flowering entomophilous crops benefits from the presence of managed and wild pollinators, who visit flowers to forage on pollen and nectar. However, management of these crops typically includes application of pesticides, the presence of which may pose a hazard for pollinators foraging in an agricultural environment. To determine the levels of potential exposure to pesticides, their presence and concentration in pollen and nectar need assessing, both within and beyond the target crop plants. We selected 11 pesticide compounds and analysed their occurrence in a crop (*Brassica napus*) and a wild plant (*Rubus fruticosus* agg.), which was flowering in field edges. Nectar and pollen from both plants were collected from five spring and five winter sown *B. napus* fields in Ireland, and were tested for pesticide residues, using QuEChERS and Liquid Chromatography tandem mass spectrometry (LC-MS/MS). Pesticide residues were detected in plant pollen and nectar of both plants. Most detections were from fields with no recorded application of the respective compounds in that year, but higher concentrations were observed in recently treated fields. Overall, more residues were detected in *B. napus* pollen and nectar than in the wild plant, and *B. napus* pollen had the highest mean concentration of residues. The most frequently detected compounds were fungicides, and all matrices were contaminated with at least three compounds. The most common compound mixture was comprised of the fungicides azoxystrobin, boscalid, and the neonicotinoid insecticide clothianidin, which was not recently applied on the fields. Our results indicate that persistent compounds like the neonicotinoids, should be continuously monitored for their presence and fate in the field environment. The toxicological evaluation of the compound mixtures identified in the present study should be performed, to determine their impacts on foraging insects that may be exposed to them.

3.2. Introduction

Insect pollination (i.e., the transfer of pollen from the male flower parts to the female, within or between flowers by foraging insects), is a key ecosystem service, providing a range of benefits to global food production and human welfare (Garibaldi et al., 2022; Fisher et al., 2009). Animal pollinators benefit the production of 75% of the globally most common food crop species, are responsible for 35% of world's crop production (Klein et al., 2007), and contribute \$235 billion -

\$577 billion to world's agriculture (IPBES, 2016). Even in partially wind-pollinated crop species like oilseed rape (OSR, *Brassica napus* L.), bees' presence can increase the average seed yield, the oil content, and improve the seed pod characteristics (e.g., weight and set) (Bartomeus et al., 2014; Garratt et al., 2014; Jauker et al., 2012; Perrot et al., 2018). Such mass flowering crops can be very attractive to pollinators (Beyer et al., 2021; Silva et al., 2021), since they flower for several weeks (Stanley et al., 2013) and produce large amounts of pollen and nectar (Holzschuh et al., 2013; Westphal et al., 2003; Bommarco et al., 2012). OSR is cultivated in several countries all over the world (Stanley et al., 2013), and is visited by various insect species (Stanley and Stout, 2014), with honey bees being the most common visitors of OSR in UK (Garratt et al., 2014), but various wild bee species being more effective pollinators of OSR (Baron et al., 2017b; Woodcock et al., 2013).

However, OSR crops tend to receive high inputs of pesticides (DAFM, 2016; Karise et al., 2017; Raimets et al., 2020) which can be detected in plant nectar and pollen of both crops (Henry et al., 2015; Pohorecka et al., 2012; Botías et al., 2015; Xu et al., 2016) and wild plant species growing on the edges of the OSR crop fields (Botías et al., 2015; David et al., 2016). Plant pollen and nectar are considered the most relevant and an important pesticide exposure route for pollinating insects, particularly bees (Sgolastra et al., 2019; Zioga et al., 2020; Chapter 2), as these are their main nutrition sources during adult and larval stages (Rortais et al., 2005; Sgolastra et al., 2019). Global declines in pollinating insects have been attributed to agricultural intensification, which involves increased pesticide application (Arena and Sgolastra, 2014; Goulson et al., 2015; Dudley and Alexander, 2017; Rundlöf et al., 2015; Sánchez-Bayo and Wyckhuys, 2019; Williams et al., 2015). In a recent review of the pesticide residues detected in plant pollen and nectar (Zioga et al., 2020; Chapter 2), the importance of quantifying the concentrations and identifying the mixtures (Sgolastra et al., 2017, 2020), of pesticide active substances contaminating pollen and nectar of both crops and wildflowers growing nearby, was highlighted. There is a paucity of information on how various pesticide compounds behave in different climates and landscapes worldwide, or even on the same field over time following their application (Sgolastra et al., 2020), and an urgent need to expand the range of compounds evaluated in plant pollen and nectar pesticide residue studies, beyond that of neonicotinoids in particular, and insecticides in general (Kyriakopoulou et al., 2017; Zioga et al., 2020; Chapter 2).

In the present study, we wanted to evaluate whether 11 compounds from different pesticide categories (fungicides, herbicides, and insecticides) contaminate pollen and nectar of OSR crops and wild plants in Ireland, and whether their presence may pose a hazard for bees during the period that they are actively foraging on OSR crops (March until July). For this, we chose winter

and spring sown OSR because a) OSR is proposed by the existing literature as a suitable crop for pesticide residue studies, b) it is the most abundant entomophilous crop in Ireland, c) it is very attractive to pollinators, d) it has a relatively long flowering period (~ 20 days), and e) due to different sowing times it flowers both in spring and summer (DAFM, 2016; Pohorecka et al., 2012; Stanley and Stout, 2013; Stanley et al., 2013; Zioga et al., 2020; Chapter 2). Blackberry (BAB, *Rubus fruticosus* agg.) was chosen as wild plant species because a) it has been evaluated in previous residue studies, b) it is an abundant wild flowering species on the margins of Irish crop fields (Botías et al., 2015; David et al., 2015), c) it is a valuable source of nectar and pollen for pollinators, and d) it has a long flowering period (~ three months) (Kavanagh, 2019; Russo et al., 2022; Wignall et al., 2020; Zioga et al., 2020; Chapter 2). The potential risk that these pesticide residues in plant pollen and nectar may pose to honey bees was then evaluated by calculating Risk Quotients.

3.3. Materials and methods

3.3.1. Field locations

In February 2019, ten conventional fields (i.e., farming that relies on chemical intervention to control pests and weeds and to provide plant nutrition) of winter sown (WOR, five fields) and spring planted (SOR, five fields) oilseed rape (OSR) were randomly selected in the East and Southeast Ireland (Figure 3.1). For each field, all information related to pesticide applications during the crop season (e.g., products applied, date and rate of application etc), along with general information regarding the crop (e.g., crop variety, sowing date etc.) was compiled by interviewing the farmers (see Appendix B Tables B1 and B2).

The compounds selected for evaluation were three fungicides commonly applied on OSR crops in Ireland (azoxystrobin, boscalid, and prothioconazole), one herbicide applied on cereals used in rotation with OSR on the same fields (fluroxypyr), the herbicide glyphosate and its metabolite aminomethylphosphonic acid (AMPA) (DAFM, 2016), and five neonicotinoid insecticides (acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam) that have not been recently applied on the fields but may have long half-lives in the environment (e.g., clothianidin) (PPDB, 2022a) and some are toxic to bees (e.g., clothianidin, imidacloprid and thiamethoxam) (Wood and Goulson, 2017). Each compound chemical class, mode of action and status according to the EC regulation 1107/2009 (European Commission, 2009b) is recorded based on the University of Hertfordshire database (PPDB, 2022a) and shown in Table 3.1.

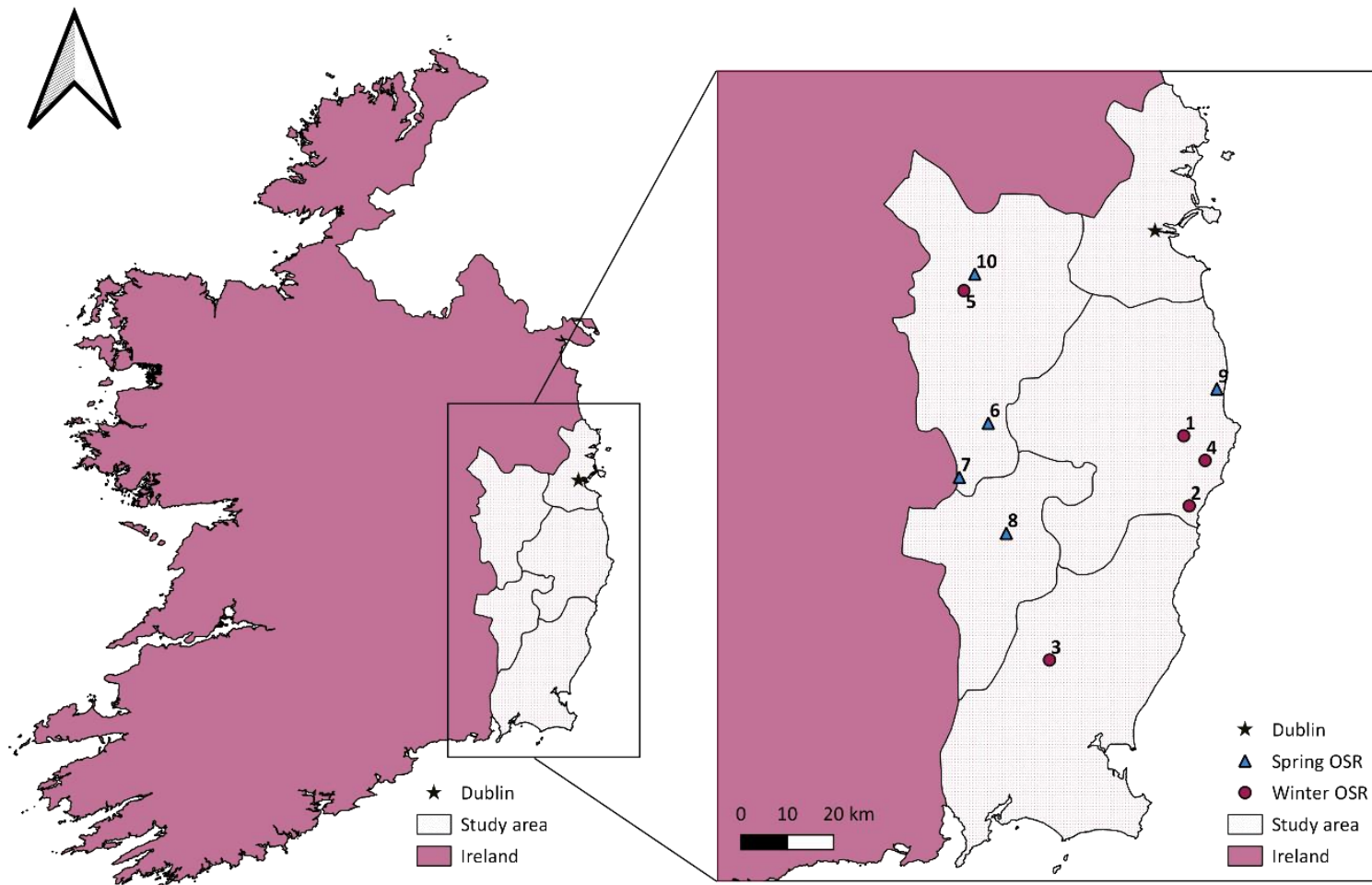


Figure 3.1. Map of the Republic of Ireland, the study area and location of the ten oilseed rape (OSR) fields, divided in five fields of spring and five fields of winter varieties.

Table 3.1. The nine pesticide compounds evaluated, their chemical class, their mode of action, their current status and expiry date according to the European regulation 1107/2009.

			EC regulation 1107/2009	
Compound	Chemical class	Mode of action	Status	Expiry date
Neonicotinoids				
Acetamiprid	Pyridylmethylamine	Nicotinic acetylcholine receptor (nAChR) competitive modulator	Approved	28/02/2033
Clothianidin	Nitroguanidine, Thiazole,		Not approved	Expired
Imidacloprid	Chloronicotine, Guanidine		Not approved	Expired
Thiacloprid	Pyridylmethylamine, Thiazolidine		Not approved	Expired
Thiamethoxam	Nitroguanidine, Thiazole		Not approved	Expired
Herbicides				
Fluroxypyr	Pyridinecarboxylic acid	Synthetic auxin	Approved	31/12/2024
Glyphosate	Organophosphate, Phosphonoglycine	Inhibition of EPSP synthase	Approved	15/12/2022
Fungicides				
Azoxystrobin	Methoxyacrylate, Strobilurin	Respiration inhibitor	Approved	31/12/2024
Boscalid	Pyridine-carboxamide, Anilide	Succinate dehydrogenase Inhibitor	Approved	31/07/2023
Prothioconazole	Conazole	Sterol biosynthesis inhibitor	Approved	31/07/2023

3.3.2. Collection of flowers from *Brassica napus* and *Rubus fruticosus* agg. plants

Crop flowers were collected during the blooming periods of the two different OSR crop types: from 19th March to 13th April (WOR) and from 17th to 27th June (SOR). During the sample collection, OSR crops were between GS63 to GS67 of the BBCH growth stages (Meier, 2001). Flowers were also collected from wild blackberries (BAB) growing in the margins and hedges of the OSR fields (~1.5 m average distance from the crop edge) during the period from 4th to 23rd July. BAB flowers were collected from eight of the ten fields, as in the remaining two sites the crop had already started to form seeds when BAB started flowering, creating an impenetrable stand of plant stems, which made the BAB plants growing in the edges of the crop inaccessible. Evaluating the temporal distribution of pesticides applied was feasible only in three fields (2, 3 and 5) (see Appendix B Tables B1 and B2). In field 3, OSR samples were collected twice after the prothioconazole application (seven and 16 days after); in field 5, OSR sampling took place both before and within a period of 20 days after the recorded azoxystrobin application; and BAB sampling was performed twice after glyphosate application on the crops (three and five days in field 2 and two and seven days in field 5). To obtain enough pollen and nectar for chemical analysis (100 mg and 100 µl respectively), a minimum of 1000 flowers from each plant species in each field and each sampling event (pre/post pesticide application) were collected. Flowers were stored in coolers during sampling and when transferred in the lab, they were weighed and subsequently stored at -25 °C prior to pollen and nectar extraction.

3.3.3. Nectar and pollen extraction from flowers

The flowers were defrosted at room temperature. The flower petals were carefully removed from each flower for easier access to the nectar droplets located at the base of the filaments. The nectar was collected with a precision-bore glass microcapillary tube (1 µL - 5 µL, Hirschmann®). Using a micropipette, nectar was extracted from the microcapillary tubes and transferred in a labelled and pre-weighed plastic 2 mL centrifuge vial. For each sample, the final number of flowers processed was recorded and the respective amount of nectar was weighed. Sugar content (as sucrose equivalent) in OSR and BAB floral nectar was measured using a handheld refractometer and was found to be $39 \pm 1.8\%$ and $28 \pm 2.3\%$ respectively. Nectar samples were stored in the freezer at -25 °C prior to chemical analysis.

After the nectar extraction, the stamens of the flowers in both plant species were cut from the rest of the flower parts and they were incubated at 37 °C for 24 hours, to facilitate pollen release from the anthers. Then, the stamens were brushed over sieves to separate pollen from the anthers and sifted through multiple sieves of decreasing pore size (250 to 45 µm). The final

amount of pollen collected was weighed following the same process with nectar, and pollen samples were evaluated under the microscope for impurities. The pollen samples were stored in the freezer at -25 °C prior to chemical analysis. Our pollen extraction process follows the protocol of Botías et al. (2015).

3.3.4. Pesticide residue analysis

3.3.4.1. Chemicals and reagents

Certified analyte standards of thiamethoxam, thiacloprid, acetamiprid, clothianidin, imidacloprid, prothioconazole, fluroxypyr, boscalid, azoxystrobin, internal standards (IS) (clothianidin-d₃, imidacloprid-d₄, thiamethoxam-d₃, thiacloprid-d₄, acetamiprid-d₃, malathion-(diethyl-d₁₀), MCPA-(methyl-d₃)), formic acid, ammonium formate, Supel™ QuE, PSA/C18/ENVI-Carb, Millipore Millex syringe filters with hydrophobic PTFE membrane (pore size 0.22 µm and 20 mm diameter) and low-adsorption LC-MS certified vials were obtained from Sigma Aldrich, Ireland. The XBridge UPLC BEH column 4.6 × 100 mm i.d., 3.5 µm particle size using XBridge BEH C18 VanGuard Cartridge (3.5 µL 3.9 × 5 mm) and VanGuard cartridge holder were all purchased from Waters Chromatography, Ireland. All pesticide standards were > 98% compound purity and deuterated standards > 98% isotopic purity. Ultrapure water was generated using ELGA Purelab Ultra SC MK2 (ELGA, UK), and LC-MS grade acetonitrile (ACN) was obtained from Sigma-Aldrich, Ireland. Individual standard pesticide (native and deuterated) stock solutions (1 mg/mL) were prepared in ACN. An additional IS mixture of the deuterated pesticides at 1000 ng/mL was also prepared. Calibration points in H₂O/ACN (70:30) were freshly prepared from the stock solutions. All stock solutions were preserved at -25 °C in dark conditions. The glyphosate and AMPA chemical analysis protocols are described in full detail in (Zioga et al., 2022; Chapter 5).

3.3.4.2. Nectar extraction

100 µL of nectar was placed into a 15 mL PTFE centrifuge tube. A solution of H₂O/ACN (70:30) was added so that the volume of the final extract was 1 mL. The nectar sample was then homogenized using a vortex and centrifuged at 2500 rpm for 5 min. The solution was filtered through 20 mm PTFE hydrophilic syringe filters (Sigma-Aldrich) into an LC-MS vial.

3.3.4.3. Pollen extraction

Samples were extracted using a modified QuEChERS method as described by Anastassiades et al. (2003). Briefly, 100 mg were weighed in a 50 mL PTFE centrifuge tube to which 1 mL of ultrapure H₂O was added. The tube was vortexed for 1 min and then sonicated for 30 min. Then

4 mL of acidified ACN (5% formic acid) were added and the tube was vortexed for 1 min. For the partitioning, 0.5 g ammonium formate salt was added, and the sample was vortexed for 1 min. The tube was centrifuged at 2500 rpm for 2 min. The supernatant was transferred into a 15 mL PTFE centrifuge tube and mixed with the Supel™ QuE, PSA/C18/ENVI-Carb. The 15 mL tube was vortexed for 1 min and then centrifuged at 4500 rpm for 10 min. A 400 µL aliquot of the supernatant was mixed with 100 µL ACN and diluted with 500 µL acidified ultrapure water (0.2% formic acid). The solution was filtered through 20 mm PTFE hydrophilic syringe filters (Sigma-Aldrich) into an LC-MS vial.

3.3.4.4. LC-MS/MS analysis

The analysis of the nine compounds in both nectar and pollen, was performed on an Agilent 1290 Infinity II Multisampler LC System coupled to an Agilent 6470 Triple Quadrupole mass spectrometer (LC-MS/MS). Compound separation was achieved on an XBridge UPLC BEH column 4.6 × 100 mm i.d., 3.5 µm particle size, Waters) using a column oven at 30 °C and a flow rate of 0.5 mL/min. Mobile phase A consisted of 5 mM ammonium formate with 0.1% formic acid and mobile phase B comprised ACN with 0.1% formic acid. The following gradient elution programme was used: linear increase from 30 to 50% B in 5.0 min, 50 to 80% B in 3 min, 80 to 94 in 2 min, 94 to 98 in 1 min, 98 to 100 in 1 min, holding at 100% B for 2 min and returning to initial conditions at 30% B in 1 min. Total time of the LC-MS/MS analysis was 15 min. The injection volume was 10 µL. The ion spray voltage was set at 3500 V and - 3500 V for positive and negative ionization respectively. Source temperature was set at 325 °C. Nitrogen was used as a curtain gas (8 L/min), nebulizer gas (25 psi), sheath gas flow (11 L/min), and sheath gas temperature was 375 °C. Agilent Mass Hunter Workstation Data Acquisition Version 10.0 software was used to control LC-MS/MS system and for data acquisition. Quantitative and qualitative analysis was done with Agilent Mass Hunter Workstation Qualitative Analysis software version 10.0, based on two most abundant precursor ion and product ion MRM transitions, and their characteristic retention time. The compound-specific LC-MS/MS retention times (Rt), quantifying transition ions (Q) and qualifier transition ions (q) for the nine compounds analysed are shown in Appendix B Table B3.

3.3.4.5. Method validation

Validation experiment was carried out according to requirements of Document No. SANTE/12682/2019 (European Commission, 2020a) guidance to evaluate linearity, matrix effect, limit of detection and quantification, sensitivity, accuracy, and precision (see Appendix B Table B4) (European Commission, 2020a). To prepare the pollen matrix-matched calibration,

pollen from organic beekeeping was used as blank. For the nectar matrix-matched calibration, a nectar surrogate was created by mixing 6 g of glucose and 3 g of fructose in 25 mL of ultrapure water (Martel et al., 2013). Linearity was evaluated both in pure solvent and matrices, using nine-point matrix-matched calibration curves ($R^2 > 0.99$). The matrix effect of nectar and pollen extracts was evaluated by comparing the slopes of the calibration curves in pure solvent (H_2O/ACN ; 70:30) with those in the respective matrix. Method sensitivity was examined by calculating the limit of quantification (LOQ) as the minimum amount of analyte detected with a signal-to-noise ratio of ten, and the limit of detection (LOD) as the minimum amount of analyte detected with a signal-to-noise ratio of 3 (David et al., 2015). Recovery (%) and precision (RSD%) were performed by fortifying blank pollen and nectar matrix samples at three concentration levels of the test analytes, for three replicates. Recoveries between 70-120% with RSD <20% were considered satisfactory (European Commission, 2020a). Concentrations were determined using a least-square linear regression analysis of the peak area ratio versus the concentration ratio (native analyte to deuterated IS) (David et al., 2015).

3.3.4.6. Quality control

The instrument was calibrated between sample batches to monitor sensitivity changes after a batch analysis. A workup sample (i.e., standard solution) per batch of ten samples was included in the worklist of the LC-MS/MS run to ensure that no contamination occurred during the sample preparation and that there was no carryover during the injections (David et al., 2015). Three replicates per sample were analysed, and analyses were performed twice for the samples where pesticides were detected.

3.3.5. Risk evaluation

For evaluating the potential risk that the active substances detected in plant pollen and nectar pose to honey bee adult foragers and nurse bees, we estimated the acute and chronic Risk Quotients (RQs) using the BeeREX risk assessment tool suggested by US EPA as tier 1 risk assessment approach (US EPA, 2015). The respective RQ values were calculated by using the value of acute dosage that kills 50% of honey bees within a given period after first exposure (LD_{50}) and were retrieved from the University of Hertfordshire online database (PPDB, 2022a) and chronic 10-day oral NOED values (no observed effect dose) were retrieved from the US EPA OPP Pesticide Ecotoxicity Database (US EPA OPP, 2017). For nectar, the daily consumption rates are suggested to be 292 mg/bee for foragers and 140 mg/bee for in-hive workers, and for pollen the daily consumption is 9.6 mg per in-hive worker and zero for foragers (US EPA, 2014). In cases where the 10-day oral NOED data from chronic oral toxicity tests on honey bees were not

available we used $1/10^{\text{th}}$ of the oral LD_{50} value as a worst-case scenario (Rondeau and Raine, 2022; Xiao et al., 2022). Acute and chronic RQ values above the level of concern (LOC, 0.4 and 1 respectively), would imply that further assessment is needed (Thompson, 2021).

3.3.6. Statistical analysis

The concentrations of residues and the RQ values were calculated in Excel (version 2108, Microsoft®, Redmond, WA, USA). The software used for map creation was QGIS Geographic Information System (QGIS, version 3.12.1), and part of graph creation was GraphPad Prism Software (version 9.3.1). Non-parametric Mann-Whitney (M-W) U tests were used to compare the concentrations of compounds present in pollen and nectar collected from OSR flowers, to compare pollen and nectar collected from OSR flowers versus pollen and nectar from BAB flowers growing in the OSR field margins, and for pollen and nectar collected from WOR versus those collected from SOR. To perform the statistical analyses, all concentrations that were $\geq$ LOD but $<$ LOQ were assigned half of the LOQ value for each compound (Dively and Kamel, 2012; Zioga et al., 2020; Chapter 2) (see Appendix B Table B4). Concentrations below the LOD were assigned a zero value. The coefficient of variation in the concentrations of compounds found in OSR pollen and nectar, and in BAB pollen and nectar, was used to analyse the consistency in the residue levels found in these sets of samples, using non-parametric tests to compare between the variability found in OSR pollen and OSR nectar, and in OSR pollen and BAB pollen. The relationship between the concentrations of residues in nectar and pollen was assessed using a paired Generalized Linear Mixed Model approach following the method described in detail in Zioga et al. (2020) (Chapter 2). The concentration of residues in pollen ($\mu\text{g}/\text{kg}$) was fitted as the response variable. The natural log of residue concentrations in pollen ($\mu\text{g}/\text{kg}$), and the compound type, were fitted as predictor variables (fixed factors). The model accounted for non-independence of data within 'Field', and 'Species' by fitting these as random non-nested factors. The model was fitted using a Gamma error distribution, and a log link function. Analyses were conducted using R version 4.2.1 (R Core Team, 2022).

3.4. Results

3.4.1. Pesticide active substances applied on the OSR fields

All ten OSR crop fields sampled in 2019 were treated with pesticides. In total, 19 different active substances were applied, the majority of which were herbicides ($n = 11$), followed by fungicides ($n = 7$), and one molluscicide (Figure 3.2). No insecticides were applied during the year of sampling on any of the OSR fields. According to the information on pesticide applications provided by the farmers, the fungicide prothioconazole was the most applied compound both

in winter and spring fields. Comparing the two different crop types (spring and winter OSR) the WOR crops received more active substances and had a higher chemical input of both fungicides and herbicides. Of the other compounds that were the focus of the present study, boscalid and azoxystrobin were applied on one field each, while the herbicide fluroxypyr and the five neonicotinoid compounds were not applied on any of the OSR crops before or during the cropping period.

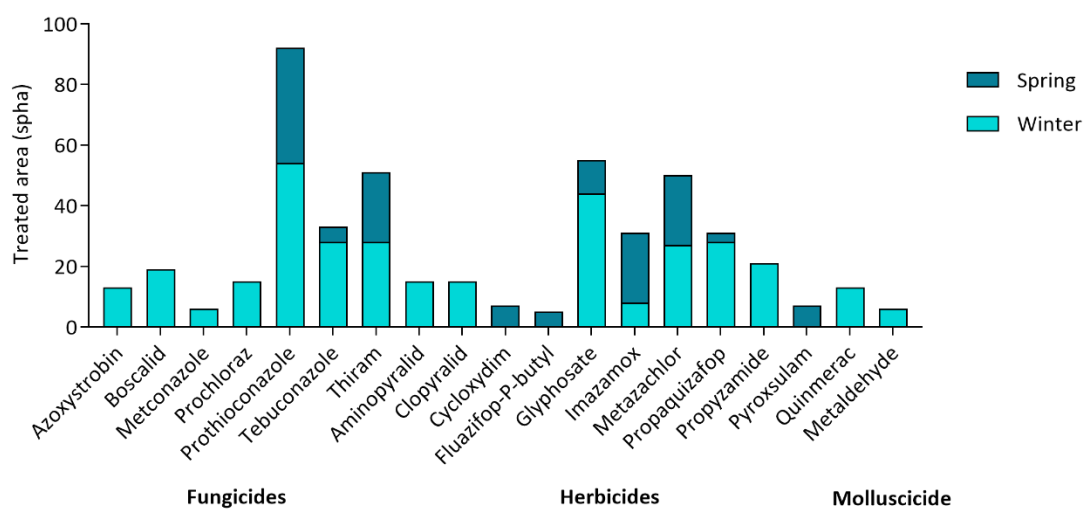


Figure 3.2. Active substances applied on the ten spring and winter oilseed rape fields sampled, their respective pesticide category - fungicides, herbicides, or molluscicides and their treated area (the total area treated with a pesticide, which includes all repeated pesticide applications, and it is measured in spray hectares).

3.4.2. Pesticide active substances occurrence on a field scale

Six out of the 11 evaluated compounds were found to be present in several of the samples analysed, even in cases where there were no reports of them having been applied on the fields (Figure 3.3). Specifically, the majority of azoxystrobin and boscalid detections and all the clothianidin and thiacloprid detections came from fields in which there were no reports of their application during the year of sampling. Conversely, the compound prothioconazole was only detected in some of the fields that it was applied to. Azoxystrobin and boscalid were detected in all ten fields, clothianidin in four fields, prothioconazole and glyphosate in three, and thiacloprid only in one field. The detected combinations of compounds that co-occurred were azoxystrobin with boscalid ($n = 10$), azoxystrobin, boscalid and clothianidin ($n = 4$), azoxystrobin with boscalid and prothioconazole ($n = 3$), azoxystrobin, boscalid and glyphosate ($n = 2$), azoxystrobin with boscalid, clothianidin and prothioconazole ($n = 1$) and azoxystrobin, boscalid, glyphosate and thiacloprid ($n = 1$) (Appendix B Figure B1). In field 5 where OSR sampling was

performed before and after azoxystrobin and boscalid application, both residues were detected in both sampling events, but the concentrations were higher in the post application event (Figure 3.3). No temporal variation of the detected compound mixtures was observed between the OSR and WOR crops, except for thiacloprid and glyphosate that were only detected in BAB from WOR fields. The compounds acetamiprid, imidacloprid, thiamethoxam, fluroxypyr, and the main metabolite of glyphosate (AMPA), were not detected in any of the matrices evaluated in the present study. None of these four compounds were reported to have been applied in any of the ten OSR fields.

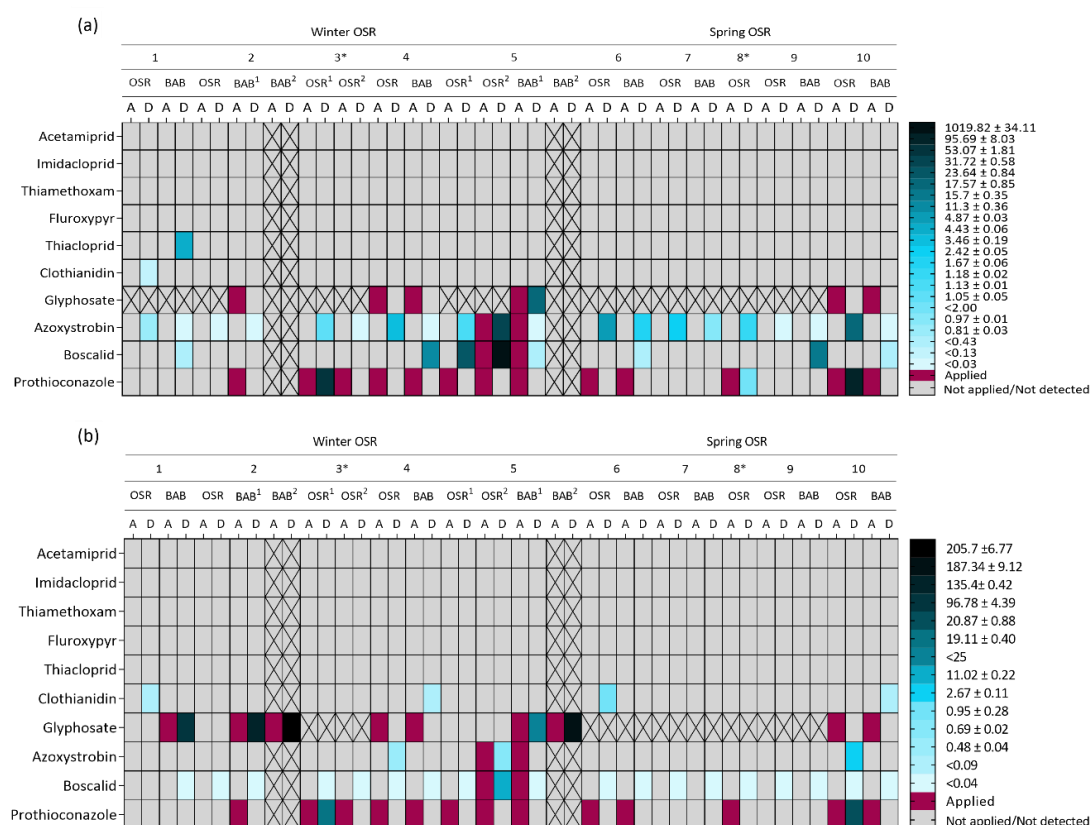


Figure 3.3. Occurrence of the nine evaluated active substances in (a) pollen and (b) nectar samples. The first column under each plant species *Brassica napus* (OSR) or *Rubus fruticosus* agg. (BAB) provides information on the application of the active substance (A). If it is coloured in red, it means that the active substance was applied on the field where the sample was collected, while a grey cell implies no application. The second column indicates whether that active substance was detected or not in the respective sample (D). The blue-black colour scale shows each compound detection in samples, while each grey cell indicates there was no detection. The concentrations are measured in µg/kg. The cells marked with an X refer to samples that were not evaluated for the respective compound.

¹Refers to the first sampling event of OSR or BAB flowers on a specific field.

²Refers to the second sampling event of OSR or BAB flowers on a specific field.

*No BAB flowers were collected from fields 3 and 8.

3.4.3. Pesticide active substances detections at individual sample scale

3.4.3.1. Compound and matrix specific detections

On a per sample basis, we recorded 67 pesticide detections (29 > LOQ). The overall most frequently detected compound was boscalid (n = 27 detections/4 applications), followed by azoxystrobin (n = 23 detections/4 applications), glyphosate (n = 6 detections/15 applications), prothioconazole (n = 5 detections/26 applications), clothianidin (n = 5 detections/0 applications) and thiacloprid (n = 1 detection/0 applications) (Figure 3.3). Of the compounds applied, glyphosate was never applied during OSR flowering, but was applied as WOR desiccant when BAB plants were flowering, azoxystrobin and boscalid were applied during OSR flowering, but no BAB plants were flowering at the time, and prothioconazole was mainly applied when OSR was not flowering (60% of applications). Individual compounds were almost equally detected in pollen and nectar (n = 34 > n = 33). OSR pollen and nectar had the same number of detections (n = 18), followed by BAB pollen (n = 16) and BAB nectar (n = 15) (Appendix B Figure B2a). The most common compounds detected in pollen were azoxystrobin (n = 20) and boscalid (n = 8), and for nectar, boscalid (n = 19) and glyphosate (n = 5), though prothioconazole was most frequently applied. Fungicides were the most common compounds detected in all matrices. Azoxystrobin was more frequently detected in pollen samples (n = 20), and it was not detected in any of the BAB nectar samples. Conversely, boscalid was more frequently detected in nectar (n = 19) than in pollen samples (n = 8). Both compounds were equally detected in BAB flowers from SOR and WOR fields, but there were more detections in WOR flowers. Both compounds were detected more frequently than they were applied. Glyphosate was mainly detected in BAB nectar (only one detection in BAB pollen), while it was not detected in OSR samples. Prothioconazole was detected equally in both WOR and SOR nectar and pollen but was not found in any of the BAB samples, and in all matrices was not detected in most sites where it was applied. Although neonicotinoids were not applied, clothianidin was detected, mainly in nectar of both crop and wild species and was also found in WOR pollen. Thiacloprid was only detected once, in BAB pollen collected from a WOR field.

3.4.3.2. Concentration specific contaminations

The number of detections and median concentrations of residues were higher in WOR than SOR flower pollen and nectar (Appendix B Figure B2a, b), which corresponded with the number of applications of pesticides being higher in WOR than SOR. However, no statistically significant

differences were observed between the concentrations of residues in OSR and WOR (M-W test, $U = 468$, $p = 0.345$). The overall concentrations of residues were significantly higher in OSR pollen than OSR nectar (M-W test, $U = 79$, $p = 0.007$) and BAB pollen residues (M-W test, $U = 86$, $p = 0.04$) (Figure 3.4). Boscalid had the highest residue recorded and the highest variability in concentrations, ranging from 0.02 to 1020 $\mu\text{g/kg}$, followed by azoxystrobin with a range of residues from 0.02 to 31.7 $\mu\text{g/kg}$ (Table 3.2). The concentrations of azoxystrobin were similarly highly variable in the matrices they were detected in, and the residue levels in OSR pollen were significantly higher than in OSR nectar and BAB nectar (M-W test, $U = 8$, $p = 0.009$; $U = 0$, $p < 0.001$ respectively) and the concentrations in BAB pollen were significantly higher to those in BAB nectar (M-W test, $U = 0$, $p < 0.001$). Boscalid residues were highly variable in both BAB and OSR pollen, and significant differences were observed between OSR nectar and OSR pollen, OSR pollen and BAB pollen, OSR pollen and BAB nectar and OSR nectar and BAB pollen residues (M-W test, $U = 11.5$, $p = 0.01$; $U = 15$, $p = 0.04$; $U = 8$, $p = 0.001$; $U = 15$, $p = 0.04$). No concentration differences between the samples were observed in the residues of clothianidin and prothioconazole in any of the matrices (see Appendix B Table B5). There was a strong positive correlation between the overall azoxystrobin residues detected in pollen and those detected in nectar ($\beta = 3.332 \pm 0.965$, $p = 0.00055$) and negative correlations were observed for the overall clothianidin residues ($\beta = -4.945 \pm 1.184$, $p = 0.00003$) and glyphosate residues in BAB flowers ($\beta = -4.07611 \pm 1.936$, 0.03529) (Figure 3.5).

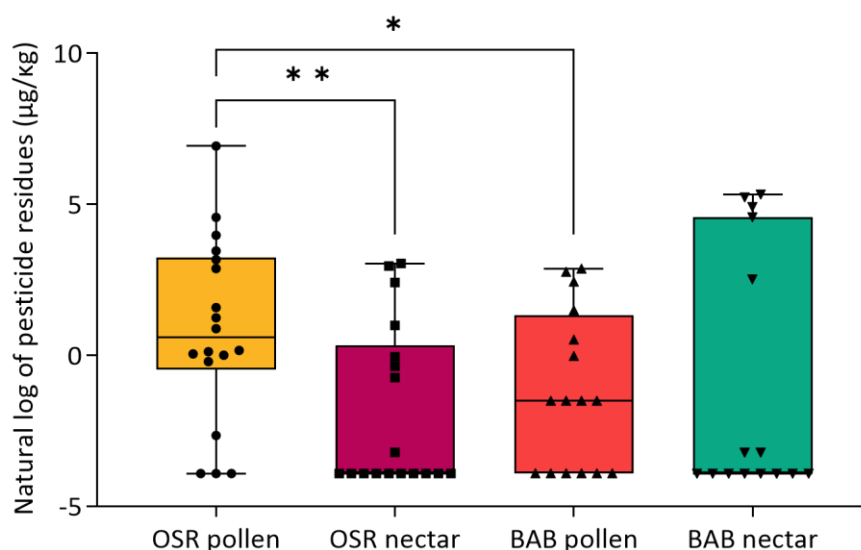


Figure 3.4. Concentration levels of pesticide residues detected in oilseed rape (OSR) and *Rubus fruticosus* (BAB) in the four different matrices. The line inside the box indicates the median value, the whiskers represent the maximum and minimum values, and the upper and lower

edges of the boxes represent the upper and lower quartiles Each point on the graph represents a pesticide detection in a sample.

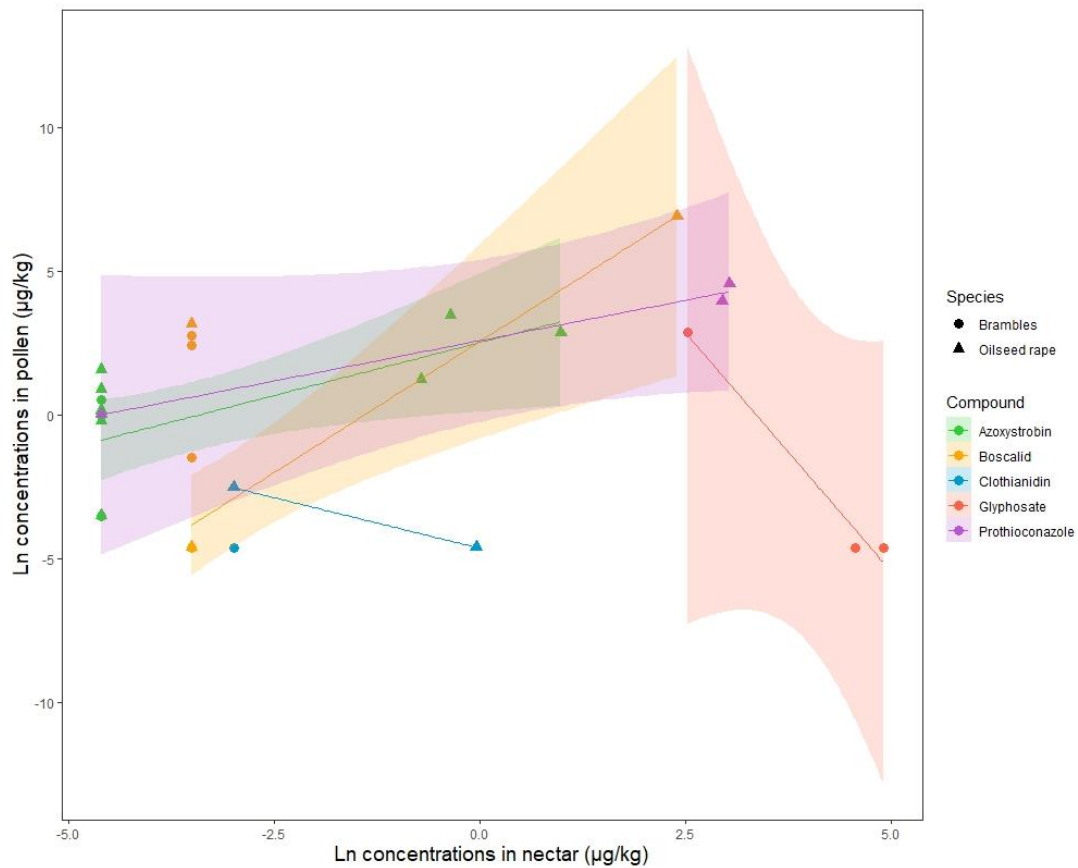


Figure 3.5. Relationship between the pesticide residues detected in nectar and pollen. Points show data from fields where both nectar and pollen were measured concurrently. Colours indicate pesticide compounds evaluated. Fitted lines show the relationship between pollen and nectar residues from the Generalized Linear Mixed Model. Shaded areas indicate the 95% confidence intervals on fitted lines.

Table 3.2. Frequency of detection, range, mean and median values of the pesticide residues detected in *Brassica napus* (OSR) and *Rubus fruticosus* agg. (BAB) pollen and nectar.

Number of samples evaluated			Insecticides		Fungicides		Herbicides	
Matrix	Insecticides and Fungicides	Glyphosate	Clothianidin	Thiacloprid	Azoxystrobin	Boscalid	Prothioconazole	Glyphosate
OSR pollen	12	2	Frequency of detection (%)	8.3	100.0	16.7	25	
			Frequency of detection >LOQ (%)	0	66.7	100	66.7	
			Range (ng/g)	<0.13	<0.03-31.72	23.64-1019.82	<2.00-95.69	
			Mean $\pm$ SD (ng/g)	-	4.94 $\pm$ 9.32	521.73 $\pm$ 704.41	49.92 $\pm$ 47.42	
			Median (ng/g)	-	1.13	521.73	53.07	
OSR nectar	12	6	Frequency of detection (%)	16.7	25	91.7	16.7	
			Frequency of detection >LOQ (%)	50	100	9.1	100	

			Range (ng/g)	<0.09-0.95	0.48-2.67	<0.04-11.02	19.11-20.87
			Mean ± SD (ng/g)	0.5 ± 0.64	1.28 ± 1.21	0.94 ± 3.17	19.99 ± 1.24
			Median (ng/g)	0.5	0.69	0.02	19.99
BAB pollen	8	4	Frequency of detection (%)	12.5	100.0	75.0	25
			Frequency of detection >LOQ (%)	100	25.0	33.3	0
			Range (ng/g)	4.43	<0.03-1.67	<0.43-15.7	<35
			Mean ± SD (ng/g)	4.43 ± 0.06	0.34 ± 0.63	4.65 ± 7.00	-
			Median (ng/g)	4.43	0.02	0.22	-
BAB nectar	8	7	Frequency of detection (%)	25.0		100	71.4
			Frequency of detection >LOQ (%)	0		0	80
			Range (ng/g)	<0.09		<0.04	<25-205.7
			Mean ± SD (ng/g)	-		-	127.54 ± 77.34
			Median (ng/g)	-		-	135.41

3.4.3.3. Temporal distribution of glyphosate, prothioconazole, azoxystrobin and boscalid

In fields 2 and 5, glyphosate was detected in BAB nectar samples when sampling was performed three and five, and two and seven days after its application on the crop respectively. In the second sampling event of both fields, the concentrations were higher than the first sampling (Figure 3.3b). In field 3, where WOR sampling was performed seven and 16 days after prothioconazole application, no residues of the compound were detected in the second sampling (Figure 3.3). In field 5, when sampling of WOR was performed prior to azoxystrobin application, 1.13 µg/kg were detected in pollen and no detections were observed in nectar, but when sampling was performed 20 days after the application, the concentrations were 31.72 µg/kg in pollen and 0.69 µg/kg in nectar. In the same field, boscalid was detected in WOR nectar (<LOQ) and pollen (23.64 µg/kg) prior to the product application, and 11.02 µg/kg and 1019.82 µg/kg seven days after the pesticide application (Figure 3.3).

3.4.4. Risk quotients

We used the maximum residues detected in plant pollen and nectar to calculate the acute and the mean residues for the chronic Risk Quotient calculation for honey bees (*Apis mellifera*) - we were limited to this species due to lack of data for LD₅₀s for other bee species (see Appendix B Table B6). Based on our results no pesticide compound was detected above the level of concern for causing acute or chronic toxicity to honey bees. By plotting the acute and chronic RQs for each plant species (see Appendix B Figure B3), we observe higher mean RQ values for residues detected in OSR.

3.5. Discussion

Several detections of selected pesticide compounds were recorded in plant pollen and nectar of both crops and non-target wild plants in agricultural fields in Ireland. Evaluating the application timing, fate, and chemical characteristics of each compound separately we identified different detection patterns. The most common compound combination was between the pesticide categories of fungicides and insecticides.

3.5.1. Pesticide applications and detections in an Irish context

When considering the treated area (i.e., the total area treated with a pesticide, which includes all repeated pesticide applications, and it is measured in spray hectares, see López-Ballesteros et al., 2022), the number of applications in each field, and the number of all individual pesticide compounds applied in OSR fields in the present study, herbicides were more widely applied than fungicides, which is in accordance with the results of the Irish Arable Crops Survey Report

(DAFM, 2016). However, no insecticides were used on the OSR crops, and the fungicide prothioconazole was applied more than the herbicide glyphosate. It was also observed that some compounds applied in WOR were not applied in SOR and vice versa. During spring time (for Northern hemisphere, from March to May), pesticide applications are typically highest, so bees are expected to be more exposed to these contaminants in this season (Murcia-Morales et al., 2021; Tong et al., 2018). Indeed, our findings show greater number and concentrations of pesticides residues in WOR (flowering in early during middle spring months) rather than spring OSR (flowering in early to middle summer months), which is in accordance with similar studies (Raimets et al., 2020). It is likely that fluroxypyr applied on crops used in rotation with OSR on the same fields, will not be translocated in OSR or BAB plants, since it was not detected in any of our samples.

3.5.2. Pesticide residue detections - a global perspective

All the compounds evaluated in the present study have been previously detected in bee related matrices like honey (El Agrebi et al., 2020a; Gawet et al., 2019; Karise et al., 2017; Muli et al., 2018; Raimets et al., 2020; Thompson et al., 2019), bee bread (Bergero et al., 2021; El Agrebi et al., 2020a; Lozano et al., 2019; Simon-Delso et al., 2014) and corbicular pollen (David et al., 2016; Favaro et al., 2019; Graham et al., 2021; Raimets et al., 2020; Tosi et al., 2018). Considering that different matrices may accumulate different pesticide compounds for various reasons (Gierer et al., 2019) and since there is a need for elucidating the primary contamination source of these compounds in space and time (Graham et al., 2021), we focused our comparison on residues detected in pollen and nectar sampled directly from plants. To put the pesticide residue results of the 11 compounds evaluated in the present study into a global perspective, we collated information on previously reported detections of these compounds in plant pollen and nectar from 21 studies (see Appendix B Figure B4). These are studies included in Zioga et al. (2020) review (Chapter 2), with additional studies that were published after 2019. Studies took place from 1999 to 2018 (published from 2001 to 2021) and they originate from the Northern hemisphere, representing three continents (Asia, Europe, and North America). The compounds glyphosate, AMPA, prothioconazole, and fluroxypyr have not been previously detected in plant pollen and nectar, presumably due to their chemical properties that make them less compatible with a multi-residue analytical approach. Analytical methodologies used in various studies to date may have underestimated the pesticide exposure and presence since the detections in the various matrices are highly dependent on the number and types of compounds analysed and the sensitivity and type of the analytical method (Toselli and Sgolastra, 2020). This makes our detections of glyphosate in BAB pollen and nectar, and

prothioconazole in OSR pollen and nectar, unique. On the other hand, the fungicides azoxystrobin and boscalid were previously detected only in plant pollen, whereas we detected both in pollen and nectar.

3.5.2.1. Fungicides

Prothioconazole residues were only detected in OSR crop pollen and nectar when the compound was applied within a seven-day period from sampling. This is in accordance with the results of Wallner (2009), where prothioconazole was detected in honey bee collected nectar in decreasing concentrations until it was not detected seven days after its application to the OSR crop. No prothioconazole residues were detected in BAB pollen or nectar, in OSR crops when the compound was not applied, or when it was applied more than 16 days prior to sampling (80% of the sprayed fields). Based on our results, and due to its low solubility in water, prothioconazole is not likely to translocate from soil to pollen and nectar (Long and Krupke, 2016).

Widespread detections of azoxystrobin and boscalid were observed not only in pollen, but also in nectar from both crop and non-target plants. Azoxystrobin concentrations in our study were higher in OSR than in BAB plants, and much lower than the respective concentrations detected in the United States (Bloom et al., 2021). The latter could be attributed to the different crops evaluated (Brassicaceae versus Cucurbitaceae) and/or different application characteristics (e.g., application rate, timing in relation to flowering etc.) (Gierer et al., 2019), which are frequently missing from pesticide residue studies for bee related matrices (Zioga et al., 2020; Chapter 2). Most observations of azoxystrobin were recorded in pollen matrices, whereas the opposite scenario applies for boscalid. It is suggested that hydrophobic interactions between the pesticide and lipophilic sites of pollen are responsible for the sorption process (Campos et al., 2008). The octanol-water partition coefficients for these compounds are similar (2.50 and 2.96 respectively), implying similar lipophilicity for both compounds (PPDB, 2022a). To predict pesticide mobility in plants, we need to also consider the dissociation constant of the compounds (pKa), which is higher in boscalid (14.3) than azoxystrobin (1.9) (PPDB, 2022a). Decreasing pKa is associated with increase in plant systemicity. Hence, according to the Bromilow and Chamberlain (1991) model for the prediction of pesticide mobility in plants, azoxystrobin appears to be more mobile than boscalid. However, more research is required to investigate potential relationships between the physicochemical characteristics of an active substance to the translocation to pollen or nectar and to the fate and dissipation after the application (Crenna et al., 2020; Gierer et al., 2019). It can also be hypothesised that boscalid

dissipates faster in pollen than in nectar (Choudhary and Sharma, 2008), and/or the fact that with our analytical method, boscalid detection limit was lower for nectar than for pollen. Pollen residue concentrations of boscalid in our study were much higher than those reported from Canada and United Kingdom (David et al., 2015, 2016; Willis Chan et al., 2019). This could be attributed to the fact that our sampling took place relatively close to the application time of the compound.

Both azoxystrobin and boscalid were also detected in OSR and BAB pollen and nectar of fields where they were not intentionally applied, most likely due to translocation from soil residues from past pesticide applications (Riedo et al., 2022). The persistence in soil of these compounds (DT_{50} 8.6 and 10.3 months respectively) (PPDB, 2022a) could explain the low concentrations detected ($\leq$ LOQ). However, higher concentrations were observed especially after another fungicide was applied on the same fields. Upon discussion with the farmers, it was noticed that it was a common practice to not wash the tank in between applications of products belonging to the same pesticide category (e.g., fungicides). This could partially explain their detections in fields where they were not intentionally applied.

A positive relationship was observed between the fungicide residues detected in pollen and nectar, especially for azoxystrobin residues and this in line with previous findings (Botías et al., 2015; Kyriakopoulou et al., 2017; Zioga et al., 2020; Chapter 2).

3.5.2.2. *Glyphosate and AMPA*

In our study, glyphosate was applied as desiccant on the OSR crops and detected in the non-target BAB pollen and nectar, but not in OSR crops. The off-target contamination of flower tissues with high concentrations of glyphosate (up to 1.25 mg/kg) has been previously reported (Cebotari et al., 2018), and should not be overlooked. We detected glyphosate in higher concentrations in plant nectar than in pollen as long as seven days after its application, which is contradictory to the results of Thompson et al. (2014) where pollen and nectar collected by honey bees foraging on directly sprayed plants was evaluated, and concentrations in pollen were 10 times higher than in nectar. The difference in the pattern of the detected concentrations could be a result of the different matrix evaluated, the different plant structure (Gierer et al., 2019), or the different contamination pathway (direct spray versus spray drift or soil translocation) (Zioga et al., 2022; Chapter 5). Future work should include a temporal investigation of the pesticide's fate over multiple days to elucidate the number of days it is detected in plant pollen and nectar and how the concentrations behave over time (Zioga et al.,

2022; Chapter 5). The metabolite AMPA was not detected in any of the evaluated samples in our study.

3.5.2.3. Neonicotinoids

Various neonicotinoid residues have been recorded in the literature over the years. Neonicotinoid insecticides are still legally applied globally, but the European Union has taken measures that altered the application status of these compounds over the last decade: in 2013 the use of clothianidin, imidacloprid and thiamethoxam as seed treatments was restricted (European Commission, 2013a), in 2018 their outdoor use was banned (EFSA, 2018a, b, c), and their approvals expired on 31 January 2019, 30 April 2019 and 1 December 2020. Thiacloprid's approval was withdrawn on 3 February 2020, while acetamiprid is still approved for use (European Commission, 2022a). Recently, the European Court of Justice confirmed that Member States will no longer be allowed to grant neonicotinoid derogations (ECJ, 2023).

Despite several reported detections in other bee related matrices (El Agrebi et al., 2020b; Erban et al., 2019; Favaro et al., 2019; Friedle et al., 2021; Gawel et al., 2019; Kaila et al., 2022; Šlachta et al., 2020; Végh et al., 2022), there is scarce information on studies showing the neonicotinoid contamination of pollen and nectar from crop and non-target plants after the European Commission regulation amendments for those compounds (e.g., existing literature provides information on succeeding crops, see Thompson et al., 2022). In our study, only the compounds clothianidin and thiacloprid were detected, even though there were no reports for neonicotinoid application on the respective fields for at least three years prior to 2018 for most of the fields. Clothianidin, a metabolite of thiamethoxam, is a very persistent compound with a half-life that can last up to ~3 years and that could explain its presence in our samples (PPDB, 2022a). Clothianidin was mainly detected in OSR rather than BAB plants, a pattern that has been observed before in a similar study (Botías et al., 2016). The residues of clothianidin detected in the present study were lower than those reported for oilseed rape in similar studies (e.g., Botías et al., 2015; David et al., 2016) and this in accordance with the study of Thompson et al. (2022), where residues were detected in pollen and nectar of succeeding crops several years after neonicotinoid use. Clothianidin was mainly detected in nectar, and we observed a strong negative correlation between nectar and pollen residues. This is in contrast with the results of Zioga et al. (2020) (Chapter 2), who found a positive relationship. This could be attributed to the fact that during the studies included in the 2020 review there was a more recent application of clothianidin on the fields, as opposed to our study, where clothianidin has not been applied for years. More research could elucidate whether clothianidin residues tend to appear more in

nectar than pollen. The concentrations of clothianidin detected in both pollen and nectar in our study were much lower than those reported for Europe in previous studies (Botías et al., 2015; David et al., 2015, 2016; Thompson et al., 2016; Pohorecka et al., 2012), and both our maximum and mean clothianidin concentrations detected did not pose a hazard for bees based on the RQ estimation. Thiacloprid was detected in BAB pollen in only one field at a concentration similar to the average European detections. Thiacloprid was still legally applied during the sampling period, hence the recorded detection could be a result of spray drift or run-off from adjacent fields.

3.5.3. Compound mixtures

In our study, the detection of several compound mixtures in the same matrix and the same field was more the rule rather than the exception. This is not surprising as multiple pesticide detections in floral pollen and nectar have been previously reported (Bloom et al., 2021; Bótias et al., 2015; David et al., 2015, 2016; Pohorecka et al., 2012). However, an evaluation of the number and combinations of the compounds that were co-detected highlighted some interesting observations. A minimum of three and a maximum of four compounds contaminated the evaluated matrices, while azoxystrobin and boscalid - both fungicides, seem to be the common denominators in the field detections. Fungicides were co-detected with other fungicides (azoxystrobin, boscalid and prothioconazole), insecticides (azoxystrobin, boscalid, prothioconazole, and clothianidin), herbicides (azoxystrobin, boscalid, and glyphosate), and all three categories together (azoxystrobin, boscalid, glyphosate, and thiacloprid).

Mixtures of compounds in pollen and nectar are a potential hazard for sublethal effects (Toselli and Sgolastra, 2020; Tosi et al., 2022), and contrary to what was previously thought, recent evidence shows lethal and sublethal impacts as a result of exposure to fungicides and herbicides alone or in combinations with insecticides for various bee species (Belsky and Joshi, 2020; Cullen et al., 2019; Tosi et al., 2022). However, it is important to account for the field relevant potential exposure concentration and concentration ratio when trying to calculate the synergistic relationship between pesticide compounds belonging to various categories (Belden, 2022; Belden and Brain, 2018). In our study, most pesticide detections occurred in sublethal concentrations, and only one pesticide detection reached the mg/kg level (boscalid).

More specifically, of the compounds detected in the present study, glyphosate was found to frequently cause sublethal effects at concentrations on a ppm level (Cullen et al., 2023; Tosi et al., 2022), while chronic exposure to glyphosate concentrations alone (below the spectrum of

those we detected) or in combination with neonicotinoid and fungicide compounds may induce high toxicity in winter honey bees (Almasri et al., 2020). Field realistic levels of boscalid have been shown to interact with neonicotinoid insecticides such as thiamethoxam and clothianidin, increasing their toxicity to honey bees (Peghaire et al., 2020; Tsvetkov et al., 2017). Its presence was also linked with weak honey bee colonies (Simon-Delso et al., 2014) and consumption of its commercial formulation can lead to chronic and cumulative toxicity in adult honey bees (Simon-Delso et al., 2018). Thus, the co-occurrence of boscalid with several other active substances requires further investigation. Moreover, since the presence of azole fungicides is known to have synergistic activity with neonicotinoid insecticides (Belden, 2022), we would recommend evaluating whether the relative ratio of prothioconazole and clothianidin residues and the absolute residue of clothianidin are likely to be of concern. Azoxystrobin can act as synergist with other pesticide compounds (van Dyke et al., 2018), and alone has shown weak toxicity to honey bees (Barascou et al., 2022), induced gene transcriptional alterations (Christen et al., 2019) and histopathological and cytotoxic changes in the midgut of honey bees (Serra et al., 2023), at concentrations higher to those detected in the present study.

Even without potential synergistic effects with other pesticides, many studies report lethal and sublethal effects of clothianidin to various bee species and life stages (Charvet et al., 2004; Colgan et al., 2019; López et al., 2017; Williams et al., 2015; Wood et al., 2018), in some cases at concentrations similar to those detected in our study (e.g., 0.96 µg/kg). In our study we found several clothianidin detections, the application of which dates at least three years back in time. Since various bee species can react differently to exposure to clothianidin (Rundlöf et al., 2015), clothianidin and its co-occurred compounds should be monitored and assessed for both lethal and sublethal effects on various bee species and not just honey bees. A similar spectrum of impacts has also been reported for thiacloprid. By itself, it induces behavioural sublethal effects in bees affecting foraging, homing success and navigation (Fent et al., 2020; Tison et al., 2016), causes adverse effects to their immune system (Brandt et al., 2016) and impacts colony development of bumble bees under field conditions (Ellis et al., 2017), at much higher concentrations than those we detected. It also shows synergistic activity when co-detected with azole fungicides (Johnson, 2015; Iwasa et al., 2004; Schmuck et al., 2003; Wernecke et al., 2019), and exhibits sublethal effects (Vanderhaegen, 2017). Even though it is not applied anymore in a European context, it is still in use in other parts of the world, hence its co-occurrences with other pesticides in those areas should be further evaluated.

3.6. Conclusions

In our study we found several pesticide compound detections in the main source of pollinators' food: plant pollen and nectar, though these detections did not correspond with the pesticides recently applied in most cases. Residues were detected not only in matrices of the OSR crops, but also in nearby growing non-target wild plants. Higher concentrations were observed in the OSR crops for all compounds except for glyphosate and thiacloprid that were only detected in BAB pollen and nectar. We show that during springtime - a crucial time of the year for bees, the latter can be exposed to high concentrations and a variety of chemical compounds through their food. The RQ thresholds for honey bees were not exceeded for any of the compounds evaluated in the present study. The compounds azoxystrobin, boscalid and clothianidin co-occurred in several fields, illustrating that in field conditions bees can be simultaneously exposed to combinations of pesticides from different chemical groups that are known to act synergistically, with negative impact on bee health. However, fluroxypyr applied in rotation crops was not detected and prothioconazole seems to not present a chronic toxicity concern. The persistence of the neonicotinoid insecticide clothianidin in plant pollen and nectar several years after its outdoor ban in Europe, and since its actual application on the fields, is of concern, and suggests that continuous monitoring of neonicotinoid presence and fate in the field environment is warranted.

Chapter 4

Assessing potential bee exposure to pesticide residues in pollen from crop flowers, and bee collected pollen



To be submitted in Science of The Total Environment as: Zioga, E., White, B., Stout, J.C. Bumble bees may be exposed to different pesticides than honey bees under the same agricultural conditions.

4. Assessing potential bee exposure to pesticide residues in pollen from crop flowers, and bee collected pollen

4.1. Abstract

In an agricultural environment, where crops are treated with pesticides, bees are likely to be exposed to a range of chemical compounds in a variety of ways. The extent to which different bee species are affected by these chemicals, depends to a large extent on the concentrations and type of exposure. We quantified the presence of selected pesticide compounds in the pollen of two different entomophilous crops (*Brassica napus* L. and *Vicia faba* L.) from 12 sites in Ireland and compared our results with the pollen loads of honey bees and bumble bees actively foraging on those crops in those same sites. Detections were compound specific, and the timing of pesticide application in relation to sampling influenced the final residue contamination levels. Most detections originated from compounds that were not recently applied on the fields, and samples from *B. napus* fields were more contaminated compared to those from *V. faba* fields. Crop pollen was contaminated only with fungicides, honey bee pollen loads contained mainly fungicides, while more insecticides were detected in bumble bee pollen loads. The highest number of compounds and most detections were observed in bumble bee pollen loads, where notably, all five neonicotinoids assessed (acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam) were detected despite the European ban and no recent application of these compounds on the fields where samples were collected. The concentrations of neonicotinoid insecticides were positively correlated with the number of wild plant species present in the bumble bee pollen samples, and imidacloprid detected in bee pollen exceeded the Risk Quotient threshold for chronic toxicity. The compounds azoxystrobin, boscalid and thiamethoxam formed the most common pesticide combination in pollen. Our results raise concerns about potential long-term bee exposure to multiple residues and question whether honey bees are suitable surrogates for pesticide risk assessments for all bee species.

4.2. Introduction

While visiting flowers to collect pollen and nectar, bees, and other animals, provide valuable pollination services to both crops and wild plants (Klein et al., 2018; Ollerton, 2017). Although the most ecologically and economically significant agricultural pollinator was traditionally

considered the western honey bee, *Apis mellifera* L. (Hymenoptera: Apidae) (Carreck and Williams, 1998), non-*Apis* bee species (i.e., all bees other than honey bees) also contribute substantially to crop pollination (Garibaldi et al. 2013; Isaacs et al. 2017; Rader et al., 2015; Winfree et al. 2007). However, pollination services are threatened, as pollinator populations and diversity are declining on a global scale (Nieto et al., 2014; IPBES, 2019; Regan et al., 2015; Zattara and Aizen, 2021). One of the reasons for insect decline is the use of pesticides in modern agriculture (Arena and Sgolastra, 2014; Goulson et al., 2015; Dudley and Alexander, 2017; Rundlöf et al., 2015; Sánchez-Bayo and Wyckhuys, 2019; Williams et al., 2015). Pesticides are a hazard for bees and other beneficial insects, affecting their physiology, behaviour, and fitness (Desneux et al., 2007; Sanchez-Bayo and Goka, 2014).

When crops are treated with pesticides, various bee species can be exposed to multiple pesticide compounds while foraging for pollen and nectar, both from crops and wild plants flowering nearby (Botías et al., 2015; David et al., 2016; Zioga et al., 2023; Chapter 3). Bees rely on pollen and nectar as food resources to cover many of their basic biological functions (Conti et al., 2016; EFSA et al., 2022; Rortais et al., 2005; Sgolastra et al., 2019) and exposure through consumption of contaminated pollen and nectar by adults and larvae is the considered the most important exposure route for both honey bees and non-*Apis* bees. Nearly all bee larvae require a diet relatively rich in pollen, consuming substantially more than adults (Boyle et al., 2019; Sgolastra et al., 2019).

Despite the differences in life history traits (i.e., body size, sociality, flight season, voltinism, floral specialization, and nesting behaviour) that define the routes and levels of exposure and the different response to pesticide toxicity among different bee taxa (Arena and Sgolastra, 2014, Boyle et al., 2019; Brittain and Potts, 2011, Kopit and Pitts-Singer, 2018; Tasei 2002; Thompson, 2016, Stoner, 2016), honey bees are currently used as the main bee species evaluated for bee risk assessment of pesticides (Bireley et al., 2019; Boyle et al., 2019; Cham et al., 2019; EPPO/OEPP 2010; Gradish et al., 2019; Hinarejos et al., 2019). However, there were concerns raised about whether they can be a representative surrogate for all the rest, non-*Apis* bee species (Hinarejos et al., 2019; Topping et al., 2020, 2021). Additionally, it was determined that although current risk assessment procedures for honey bees are largely conservative, several routes of exposure are unique to non-*Apis* bees and warranted further investigation (Boyle et al., 2019; Gradish et al., 2019; Sgolastra et al., 2019).

Despite the progress made in recording and evaluating the importance of exposure routes to pesticides among various bee species, and that risk to other pollinators, such as bumble bees,

should be considered (Franklin and Raine, 2019; Graham et al., 2021; Stoner, 2016), there is a substantial lack of information on the exact levels and patterns of pesticide exposure, of non-*Apis* pollinators and how they compare with those experienced by honey bees under the same agricultural field conditions (Gradish et al., 2019). Bees foraging on farms during bloom are exposed to a wide range of pesticides through contaminated pollen (Graham et al., 2021), and pollen has been identified as the best matrix for evaluating the presence of pesticide residues in the environment (Chauzat et al., 2006, 2011; Zioga et al., 2020; Chapter 2) relevant for bee health (Tomé et al., 2020). Thus, to provide valuable information for a realistic bee risk assessment under typical field conditions, we sampled pollen of pesticide treated crops attractive to bee pollinators, and bee collected pollen from honey bees and bumble bees foraging simultaneously on those crops and identified and quantified the pesticide residues therein. Since the potential negative effects of pesticides on pollinating insects critically depend on the concentrations to which they are exposed (Rolke et al., 2016), we focused on recording and comparing pesticide residue data in both honey bee and bumble bee collected pollen, to explore whether the different bee species are exposed to different pesticide chemical groups (i.e., fungicides, insecticides, and herbicides), and whether there are differences in the pesticide contamination patterns and concentrations.

4.3. Materials and methods

4.3.1. Sampling locations

In May 2020, six fields growing spring planted oilseed rape (*Brassica napus* L., OSR) and six broad bean fields (*Vicia faba* L., BEA) were randomly selected in the Republic of Ireland (Figure 4.1). In Ireland, broad beans represent the second most common cultivated entomophilous flowering crop after oilseed rape (DAFM, 2016). All the evaluated fields employed "conventional" farming practices that rely on chemical applications of pesticides for controlling pests and weeds, hence all pesticide application information during the focal crop season (e.g., product applied, date and rate of application etc.) was recorded by interviewing the farmers (Appendix C Table C1). Pesticide products were applied in all fields except the OSR field 10. General information regarding the field (e.g., crop cultivated, variety, sowing date etc.) and the sampling (e.g., sampling date, temperature during sampling, matrix sampled etc.) are included in Appendix C Table C2.

4.3.2. Crop flower collection and pollen extraction

Crop flowers were collected during their blooming periods: from 31st May to 30th June 2020 (OSR) and from 24th May to 15th June 2020 (BEA). The flowering stage of the crops throughout the collection period was from GS63 to GS65 (OSR) and GS62 to GS65 (BEA) of the BBCH growth stages (Meier, 2001). In fields 8 and 11, sampling took place both before and shortly after the pesticide application. In field 6, where BEA was the main crop, OSR was co-flowering in between the crop lines, so sample collection was performed for both plant species. A minimum of 1000 flowers from each plant species in each field and each sampling event were collected as described in Chapter 3. Crop flowers were stored in coolers during sampling and when transferred in the lab, they were weighed and stored at -25 °C. The extraction of pollen is described in detail in Chapter 3. In short, for pollen extraction anthers were incubated at 37 °C for OSR and at 30 °C for BEA for 24 hours to facilitate pollen release, sieved over strainers and stored into plastic eppendorf tubes.

4.3.3. Collection of honey bee and bumble bee corbicular pollen

During each crop flower sampling, honey bee and bumble bee workers foraging for pollen on the evaluated crops were also collected. To acquire enough pollen for chemical analysis, a minimum of 20 honey bees and 15 bumble bees actively foraging on the crops and carrying relatively large amount of corbicular pollen were randomly collected from each field. The bumble bee species collected, included individuals of the species *Bombus lucorum* agg., *B. jonellus*, *B. lapidarius*, *B. pratorum*, *B. pascuorum*, *B. muscorum*, and *B. hortorum*. We focused on collecting corbicular pollen of actively foraging bees on the crop to quantify the concurrent pesticide exposure from the crop environment (de Oliveira et al., 2016; Graham et al., 2021; Kasiotis et al., 2014) that could have been directly linked to the pesticide levels present in crop pollen. The bees were caught by hand using a butterfly net during several transects along the crop area, placed in plastic vials in coolers before being transferred to the lab, where they were stored at -25 °C. Consequently, the pollen loads of each individual bee were weighed separately and then they were mixed according to the bee species they came from, the field they were collected at and their collection date for pollen identification under the light microscope. 500 grains of pollen were counted for each slide and identified based on their morphology, using a standard key (Crompton et al., 1993), pollen reference samples collected on the sampling fields, and online pollen reference collections (Chadwick et al., 2019; Martin and Harvey, 2017; PalDat, 2022; Pollen atlas, 2022; Pollen grains reference library, 2014). In most cases, pollen was identified to genus, but some pollen types were identified only to family level (e.g., Asteraceae).

Where possible, and especially in the case of crops, pollens were identified to species level (e.g., *Brassica napus* and *Vicia faba*) (Appendix C Table C3).

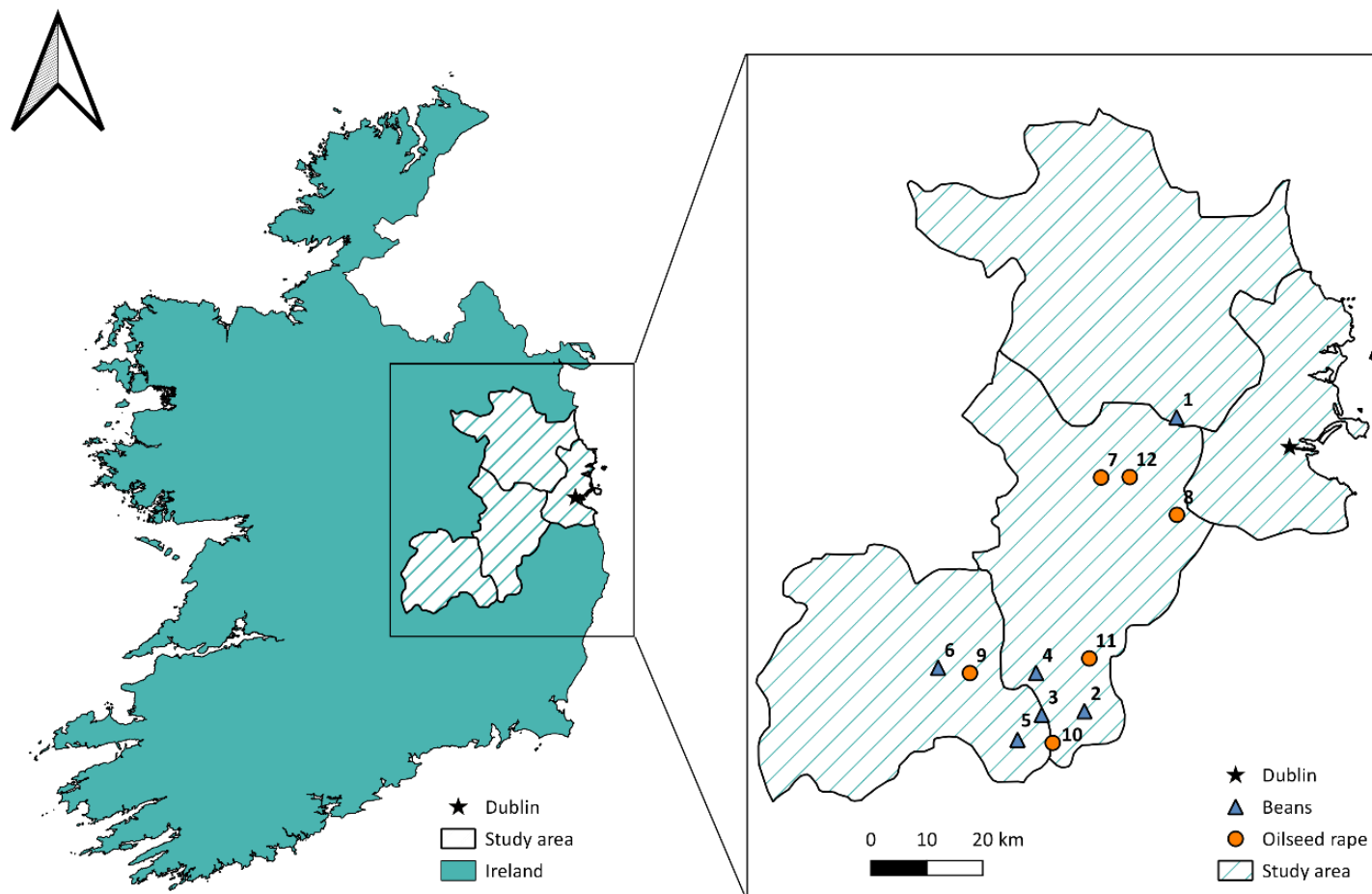


Figure 4.1. The study area in Ireland and the location of the *Brassica napus* (Oilseed rape) and *Vicia faba* (Beans) fields sampled in 2020.

4.3.4. Pesticide residue analysis

Eleven compounds were evaluated: three fungicides commonly applied on OSR and BEA crops in Ireland (azoxystrobin, boscalid, and prothioconazole), the most widely used herbicide glyphosate and its metabolite aminomethylphosphonic acid (AMPA) (DAFM, 2016), and five neonicotinoids (acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam) that have long half-lives in the environment (PPDB, 2022a) and are known to be toxic to bees (Wood and Goulson, 2017). Two different modified QuEChERS methods were developed for pollen pesticide extraction; a multiresidue method including the compounds azoxystrobin, boscalid, and prothioconazole, and the five neonicotinoids and a separate method for polar analytes (Glyphosate and AMPA). Details about the analyte standards, materials used, sample preparations, method performances and the general methodology followed, are thoroughly described in Chapter 3 and Zioga et al. (2022) (Chapter 5). For comparing our results with those of similar studies we performed a literature review on studies that evaluated pesticide residues in pollen from plants, and bee collected pollen from various bee species (e.g., honey bees, bumble bees and solitary bees). We included studies that reported analytically the pesticide residues and not those that reported the values as a range (i.e., minimum, and maximum values) and extracted all the residue information regarding our ten evaluated compounds (Appendix C Figure C1).

4.3.5. Statistical analysis

The concentrations of residues and the RQ values were calculated in Excel (version 2108, Microsoft®, Redmond, WA, USA). The software used for map creation was QGIS Geographic Information System (QGIS, version 3.12.1) and for graph creation we used GraphPad Prism Software (version 9.3.1) and R version 4.2.1 (R Core Team, 2022). The Sankey diagrams were made using the online software, Flourish (2022). Non-parametric Mann-Whitney (M-W) U tests were used to compare the concentrations of compounds present in pollen collected from OSR and BEA flowers and to compare pollen collected from three different matrices (crop flowers, honey bee and bumble bee). To perform the statistical analyses, all concentrations that were $\geq$ LOD but $<$ LOQ were assigned half of the LOQ value for each compound (Dively and Kamel, 2012; Zioga et al., 2020; Chapter 5). Concentrations $<$ LOD were assigned a zero value. To compare contamination levels between crop, honey bee and bumble bee pollen types, we ran generalized linear mixed models (GLMMs) with the pesticide concentrations detected as response variables, using a gamma distribution in R version 4.2.1 (R Core Team, 2022) as fully described in the model of Zioga et al. (2020) (Chapter 2). The type of crop (OSR or BEA) was set as fixed effect and the field as a random effect.

We performed Principal Component Analyses (PCAs) for honey bee (HNB) and bumble bee (BBB) corbicular pollen residues. To evaluate the relationship between the neonicotinoid concentrations and the number of wild plants in bumble bee pollen we ran a GLMM with the neonicotinoid concentrations detected as response variable, using a gamma distribution. The compound was set as fixed effect and the field as a random effect.

4.3.6. Risk Quotient evaluation

The acute and chronic Risk Quotients (RQs) of the detected active substances for honey bee adult nurse bees were estimated using the BeeREX risk assessment tool (US EPA, 2015) suggested by US EPA as a Tier 1 risk assessment approach, using a daily pollen consumption of 9.6 mg per in-hive worker bee (US EPA, 2014). The LD₅₀ values were retrieved from the University of Hertfordshire online database (PPDB, 2022) and chronic 10-day oral NOED (no observed effect dose) values were retrieved from the US EPA OPP Pesticide Ecotoxicity Database (US EPA OPP, 2017). In cases where the NOED data from chronic oral toxicity tests on adult honey bees were not available, we used 1/10th of the oral LDD₅₀ value (Zioga et al., 2023) (Chapter 3). Further assessment would be needed if acute and chronic RQ values are above the level of concern (LOC), which is 0.4 and 1 respectively (Thompson, 2021). It must be noted that this model could underestimate the risk since sub-lethal or synergistic effects are not considered.

4.4. Results

4.4.1. Pesticide active substances applied on the crops

As reported by the farmers, a total of 16 active substances were applied on the evaluated crops in 2020 (Figure 4.2). The majority were herbicides (n = 8), followed by fungicides (n = 6), one insecticide and one molluscicide. BEA crops received more pesticide compounds than OSR, and the herbicide propaquizafop was the most applied active substance. However, OSR fields received more input of the compounds that were the focus of the present study. Overall, azoxystrobin was applied on one field, prothioconazole in two, and boscalid and glyphosate in three fields each (see Appendix C Table C1). None of the five neonicotinoid compounds was applied on the crops.

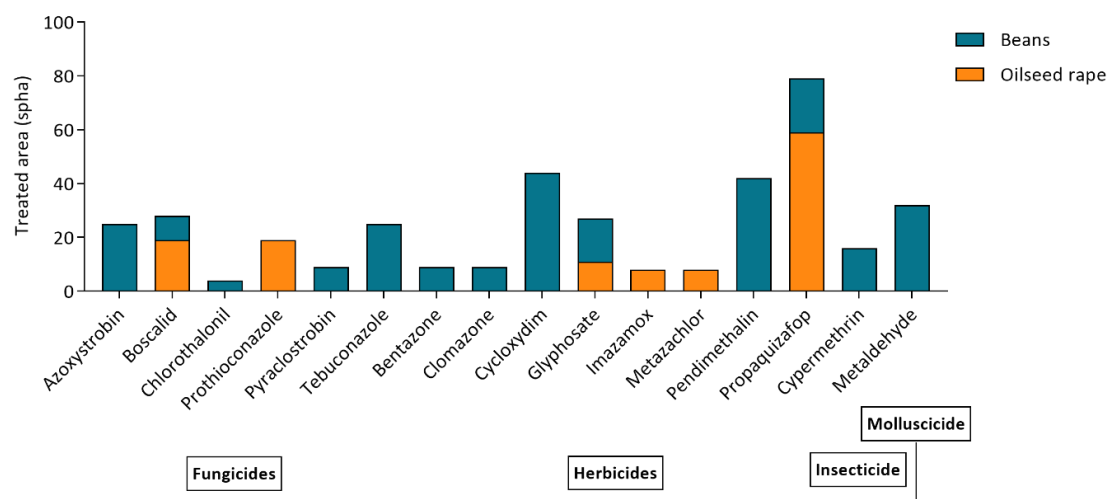


Figure 4.2. Active substances applied on the *Brassica napus* (Oilseed rape) and *Vicia faba* (Beans) fields, their respective pesticide category - fungicides, herbicides, insecticides, molluscicides, and their treated area.

4.4.2. Pesticide active substances occurrence on a field scale

Seven out of the ten evaluated compounds were found to be present in the samples analysed, even in cases where there were no reports of them having been applied on the fields (Figure 4.3). Specifically, all the azoxystrobin and neonicotinoid detections came from fields in which there were no reports of their application during the year of sampling. Conversely, the compounds prothioconazole and glyphosate were not detected in any of the fields that they were applied to. Boscalid was detected in ten fields, azoxystrobin was detected in seven fields, thiamethoxam was detected in eight fields, acetamiprid, clothianidin, and thiacloprid in two fields each, and imidacloprid only in one field. The detected combinations of compounds that co-occurred were azoxystrobin with boscalid ($n = 8$), azoxystrobin, boscalid with thiamethoxam ($n = 7$), azoxystrobin with thiamethoxam ($n = 6$), azoxystrobin, boscalid, thiamethoxam, acetamiprid, thiacloprid and clothianidin ($n = 2$), and all the seven compounds together ($n = 1$) (Appendix C Figure C2). In fields 8 and 11, where OSR sampling was performed before and after boscalid application, residues were detected in both sampling events, but the concentrations were higher in the post application event (Figure 4.3). In field 1, where sampling was performed one day after the azoxystrobin application, no residues were detected in any of the samples collected on that field. The compounds prothioconazole, glyphosate, and the main metabolite of glyphosate (AMPA), were not detected in any of the pollen samples evaluated in the present study.

4.4.3. Pesticide active substances detections at individual sample scale

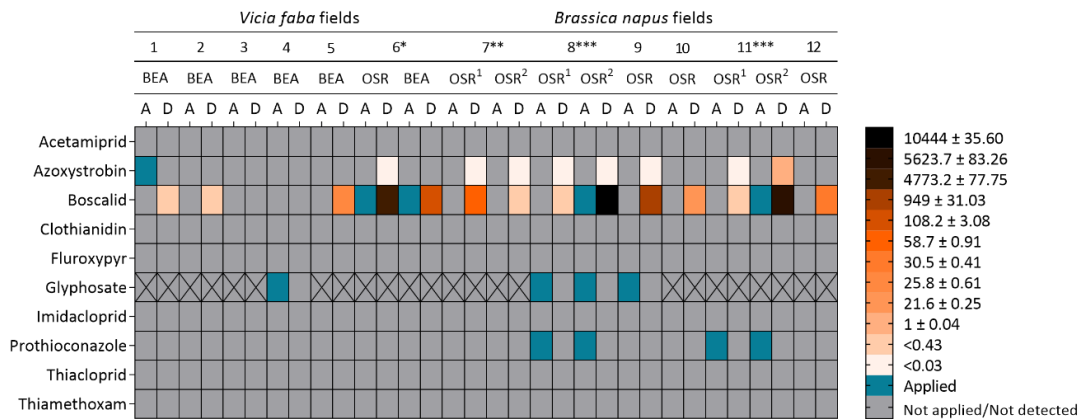
4.4.3.1. Compound and matrix specific detections

On a per sample basis, we recorded 60 pesticide detections (31 > LOQ). The overall most frequently detected compound was boscalid (n = 25 detections/12 applications), followed by azoxystrobin (n = 16 detections/3 applications), thiamethoxam (n = 12 detections/0 applications), acetamiprid, thiacloprid and clothianidin (n = 2 detections/0 applications each) and imidacloprid (n = 1 detection/0 applications) (Figure 4.3). All compounds detected, were detected more frequently than they were applied. Individual compounds were detected more frequently in crop pollen than bumble bee pollen (100% > 73%), while honey bee pollen had fewer pesticide detections (60%). All pollen types originating from OSR fields had higher numbers of detections compared to those from BEA fields (96% > 55%) (Appendix C Figure C3a). The most common compound detected in crop and honey bee pollen was boscalid (93% and 67% respectively), while for bumble bee pollen, thiamethoxam (n = 64%). Fungicides had higher numbers of detections than insecticides (83% > 34%). Specifically, crop pollen was contaminated only with fungicide compounds, honey bee pollen was contaminated mainly with fungicides (67% > 33%), while bumble bee pollen had more insecticide detections (61% > 39%). Azoxystrobin and boscalid were detected at a higher frequency in crop pollen samples (36% and 64% respectively). Although neonicotinoids were not applied on the sampled fields, all of them were detected in bumble bee pollen.

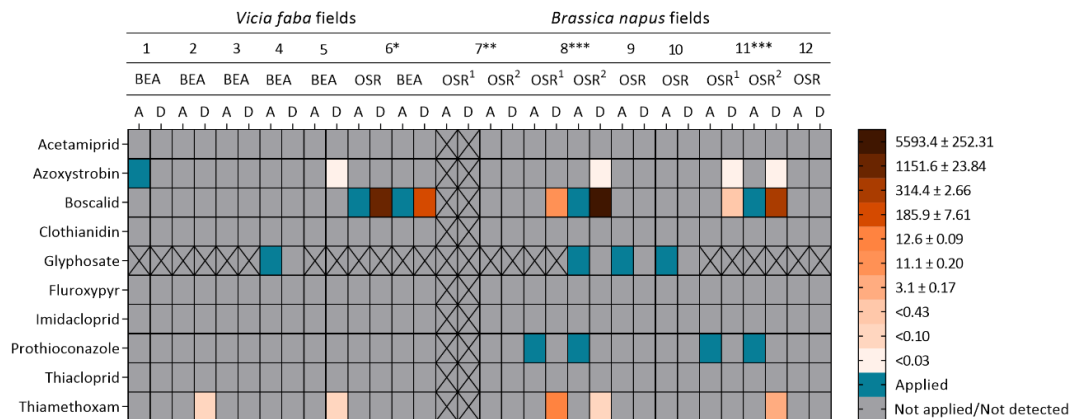
4.4.3.2. Concentration specific contaminations

The median concentrations of residues were higher in OSR than BEA pollen samples (Appendix C Figure C3a, b), which corresponds with the number of applications of pesticides evaluated in this study being higher in OSR than BEA. No significant differences were observed among the pesticide residues in the various matrices and the two crops (Figure 4.4, Appendix C Table C4). Highest mean concentrations were observed in crop pollen (1001.7 µg/kg), followed by honey bee (484.8 µg/kg) and bumble bee pollen (146.4 µg/kg). A similar pattern was observed for the average fungicides concentrations (1001.7, 725.7 and 362.0 µg/kg respectively), but no insecticides were detected in crop pollen and the average concentration of insecticides in bumble bee pollen was higher than in honey bee pollen (7.9 > 3.2 µg/kg).

(a) Crop pollen



(b) Honey bee pollen



(c) Bumble bee pollen

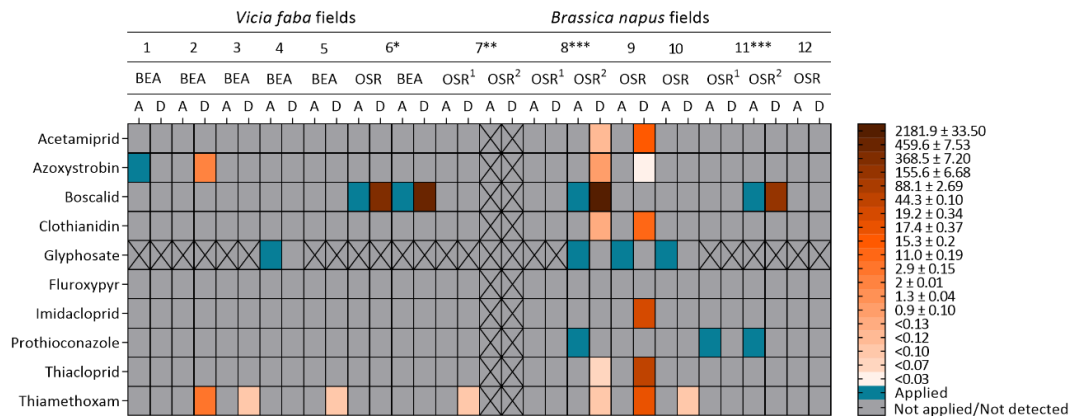


Figure 4.3. Occurrence of the evaluated active substances in a) crop, b) honey bee and c) bumble bee pollen. The first column under each crop specie provides information on the application of the active substances (A). The blue colour means that the active substance was applied on the field where the sample was collected, while a grey cell implies no application. The second column indicates whether the active substance was detected or not in the respective sample (D). The orange colour scale shows each compound detection in the different

pollen samples, while each grey cell indicates there was no detection. The cells marked with an X indicate that the respective samples were not evaluated.

*Both *V. faba* (BEA) and *B. napus* (OSR) pollen types were collected from the same field.

**In field 7, bumble bees were sampled only during the first sampling event (OSR¹) and honey bees only during the second sampling event (OSR²).

***Two sampling events were performed on those fields with OSR¹ indicating the first and OSR² the second sampling.

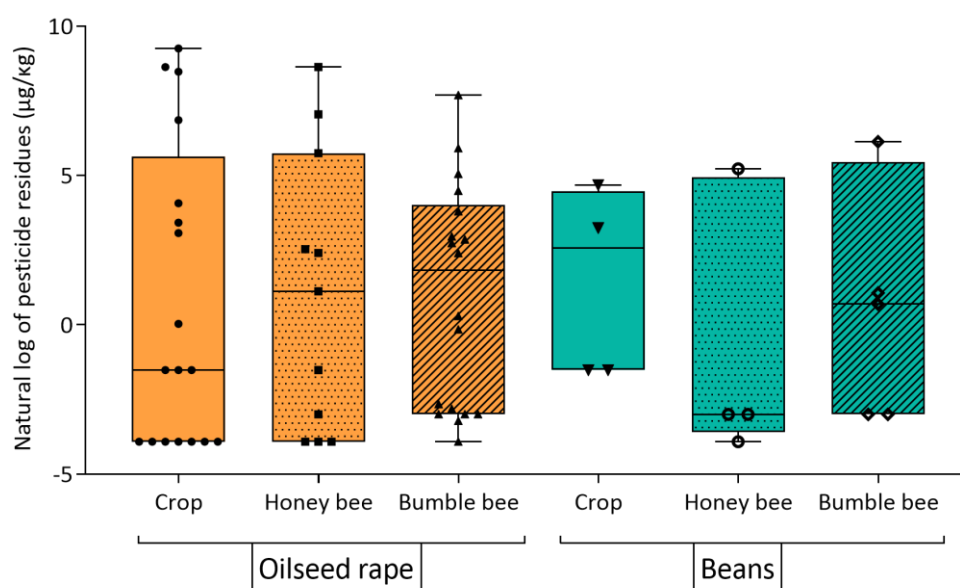


Figure 4.4. Concentrations of pesticide residues detected in the three matrices (crop, honey bee and bumble bee pollen) originating from the two crops. The line inside the box indicates the median value, the whiskers represent the maximum and minimum values, and the upper and lower edges of the boxes represent the upper and lower quartiles. Each point on the graph represents a pesticide detection in a sample.

In field 6, where OSR and BEA were co-flowering on the same field and samples were taken from both species, higher boscalid residues were recorded in OSR pollens. Boscalid had the highest residues recorded and the highest variability in concentrations, ranging from <0.43 µg/kg to 10.44 mg/kg (Table 4.1). The concentrations of azoxystrobin were highly variable in crop pollen compared to bee-collected pollen, and the residue levels in crop pollen were significantly higher than in bumble bee pollen (M-W test, $U = 5.5$, $p = 0.03$). Boscalid residues were highly variable in all matrices, and no significant differences were observed in residues among the different matrices (Appendix C Table C5). No significant differences in the concentrations were observed in the residues of thiamethoxam in honey bee and bumble bee

collected pollen. The compounds acetamiprid, clothianidin, imidacloprid and thiacloprid, were detected only in bumble bee pollen with concentrations ranging from < LOQ to 15.3, 11.0, 19.2 and 44.3 µg/kg respectively.

Strong positive correlations were observed between the concentrations of the pesticide residues of azoxystrobin, boscalid and thiamethoxam detected in crop and honey bee pollen ($\beta = 0.212 \pm 0.06$, $p = 0.0002$) (Appendix C Figure C4) and crop and bumble bee pollen ($\beta = 0.227 \pm 0.08$, $p = 0.0049$) (Appendix C Figure C5). A significant correlation was also observed between honey bee and bumble bee fungicide residues ($\beta = 0.569 \pm 0.167$, $p = 0.0007$) (Appendix C Figure C6).

4.4.4. Honey bee and bumble bee collected pollen

The majority of pollen grains in honey bee pollen from foragers in OSR and BEA crops originated from these crops ($98.8 \pm 1.51\%$ and $96.7 \pm 6.3\%$ respectively) and the same applied in the case of bumble bees, but at a slightly lower percentage for the foragers on BEA crops ($84.0 \pm 9.4\%$ and $58.0 \pm 17.5\%$ respectively). More *Bombus* spp. were observed foraging for pollen in BEA than OSR fields ($n = 7 > n=4$) (Figure 4.5 and 4.6). The species *B. jonellus*, *B. pratorum*, and *B. muscorum* were observed only in BEA fields. Overall, 20 plant families were detected in pollen loads of bees foraging in OSR crops and only 14 were detected in BEA crops. The plant family Sapindaceae (which includes horse chestnut and sycamore trees) was detected only in BEA samples, while the families Euphorbiaceae, Fagaceae (including beeches, chestnuts and oaks), Geraniaceae, Lamiaceae, Malvaceae, Poaceae and Valerianaceae were detected only in samples from OSR crops. More plant families were detected in *Bombus* spp. pollens in both crops, while the majority of different plant families was recorded in *B. lapidarius* in OSR fields ($n = 19$) and *B. lucorum* agg. in BEA fields ($n = 12$).

The number of active substances in bumble bee collected pollen increased when the percentage and the number of wild plant species in pollen loads was high (Figure 4.7). Specifically, the concentrations of insecticides were positively correlated with the number of wild plants present in the bumble bee pollen samples ($\beta = 0.646 \pm 0.308$, $p = 0.036$), but this relationship could not be verified for honey bees due to low number of unique data points (see Appendix C Figure C7). For fungicides, a negative trend was observed between the concentrations detected in both bee species pollen and the number of wild plants detected in their pollen ($\beta = -0.794 \pm 1.984$, $p = 0.079$), but it was significant only in the case of honey bees ($\beta = -4.270 \pm 1.613$, $p = 0.008$) and was mainly driven by the compound boscalid (see Appendix C Figure C8).

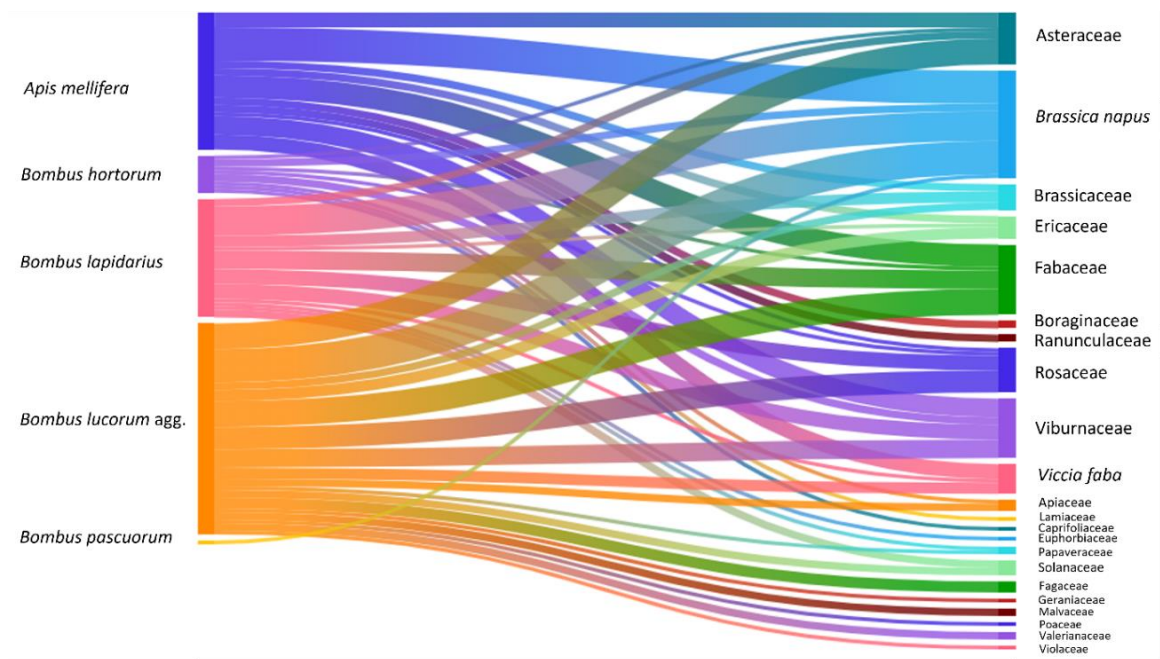


Figure 4.5. The bee species foraging on *Brassica napus* (oilseed rape, OSR) crops and the respective plant taxa detected in their corbicular pollens. The crop pollens were identified to a species level and were separated from their wild relatives. The width of the lines represents the number of bee individuals that have collected a specific plant taxon in their pollen loads.

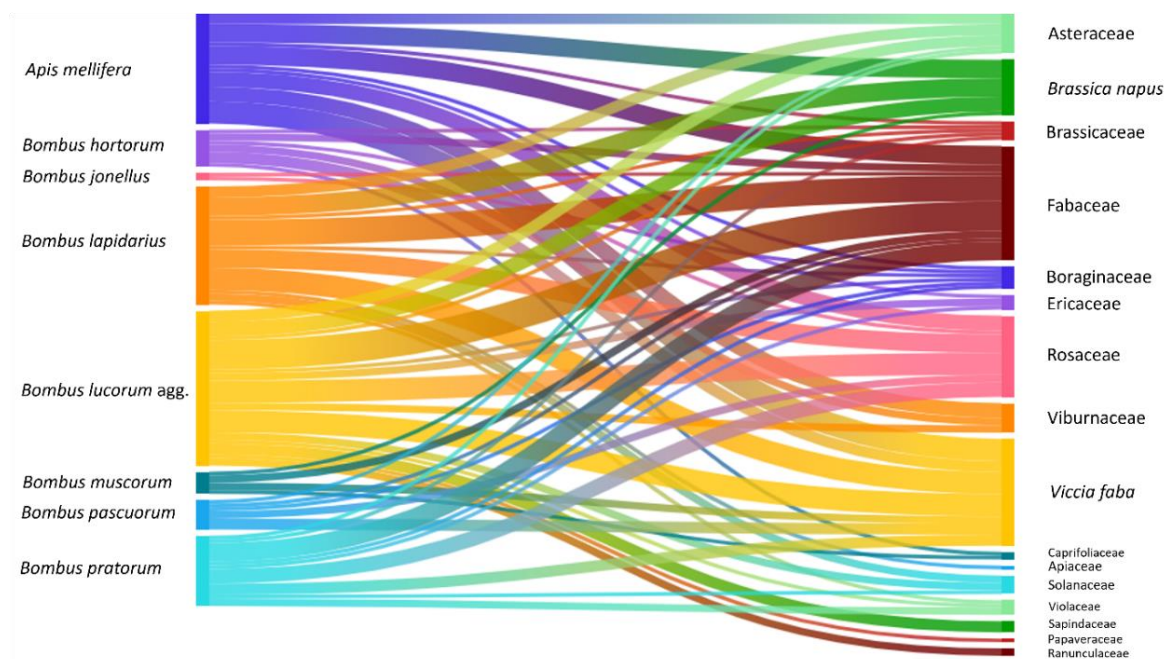


Figure 4.6. The bee species foraging on *Vicia faba* (bean, BEA) crops and the respective plant taxa detected in their corbicular pollens. The crop pollens were identified to a species level and were separated from their wild relatives. The width of the lines represents the number of bee individuals that have collected a specific plant taxon in their pollen loads.

Table 4.1. Frequency of detection, range, mean and median values of the pesticide residues detected in the six different matrices.

Crop	Pollen origin	Total number of samples	Neonicotinoids				Fungicides		
			Acetamiprid	Clothianidin	Imidacloprid	Thiacloprid	Thiamethoxam	Azoxystrobin	Boscalid
Bean	Crops	6	Frequency of detection (%)						80
			Frequency of detection >LOQ (%)						50
			Range (µg/kg)						<0.43 - 108.2
			Mean ± SD (µg/kg)						33.61 ± 51.18
			Median (µg/kg)						13.02
	Honey bee	7	Frequency of detection (%)				33.3	16.7	16.7
			Frequency of detection >LOQ (%)				0	100	100
			Range (µg/kg)				<0.10	<0.03	185.9
			Mean ± SD (µg/kg)				-	-	185.9 ± 7.62
			Median (µg/kg)				-	-	-

Oilseed rape	Bumble bee	7	Frequency of detection (%)	50	16.7	16.7
			Frequency of detection >LOQ (%)	33.3	100.0	100
			Range (µg/kg)	<0.10 - 2.90	1.99	459.6
			Mean ± SD (µg/kg)	1 ± 1.65	1.99 ± 0.02	459.6 ± 7.54
			Median (µg/kg)	0.05	-	-
	Crops	9	Frequency of detection (%)		72.7	90.9
			Frequency of detection >LOQ (%)		12.5	70
			Range (µg/kg)		<0.03 - 1.03	<0.43 - 10443.9
			Mean ± SD (µg/kg)		0.15 ± 0.36	2190.12 ± 3596.7
			Median (µg/kg)		0.02	44.6
	Honey bee	8	Frequency of detection (%)	30	30	50
			Frequency of detection >LOQ (%)	66.7	0	20
			Range (µg/kg)	<0.10 - 12.55	<0.03	<0.43 - 5593.41
			Mean ± SD (µg/kg)	5.22 ± 6.52	-	114.15 ± 2382.9

		Median (µg/kg)				3.07	-	314.43	
Bumble bee	8	Frequency of detection (%)	18.2	18.2	9.1	18.2	36.4	27.3	36.4
		Frequency of detection >LOQ (%)	50	50	100	50	25	66.7	100
		Range (µg/kg)	<0.12 - 15.32	<0.13 - 11.0	19.2	<0.07 - 44.32	<0.10 - 17.38	<0.03 - 1.34	88.13 - 2181.86
		Mean ± SD (µg/kg)	7.69 ± 10.79	5.54 ± 7.73	-	22.18 ± 31.31	4.38 ± 8.67	0.74 ± 0.67	698.51 ± 996.1
		Median (µg/kg)	7.69	5.54	-	22.18	0.05	0.85	262.03

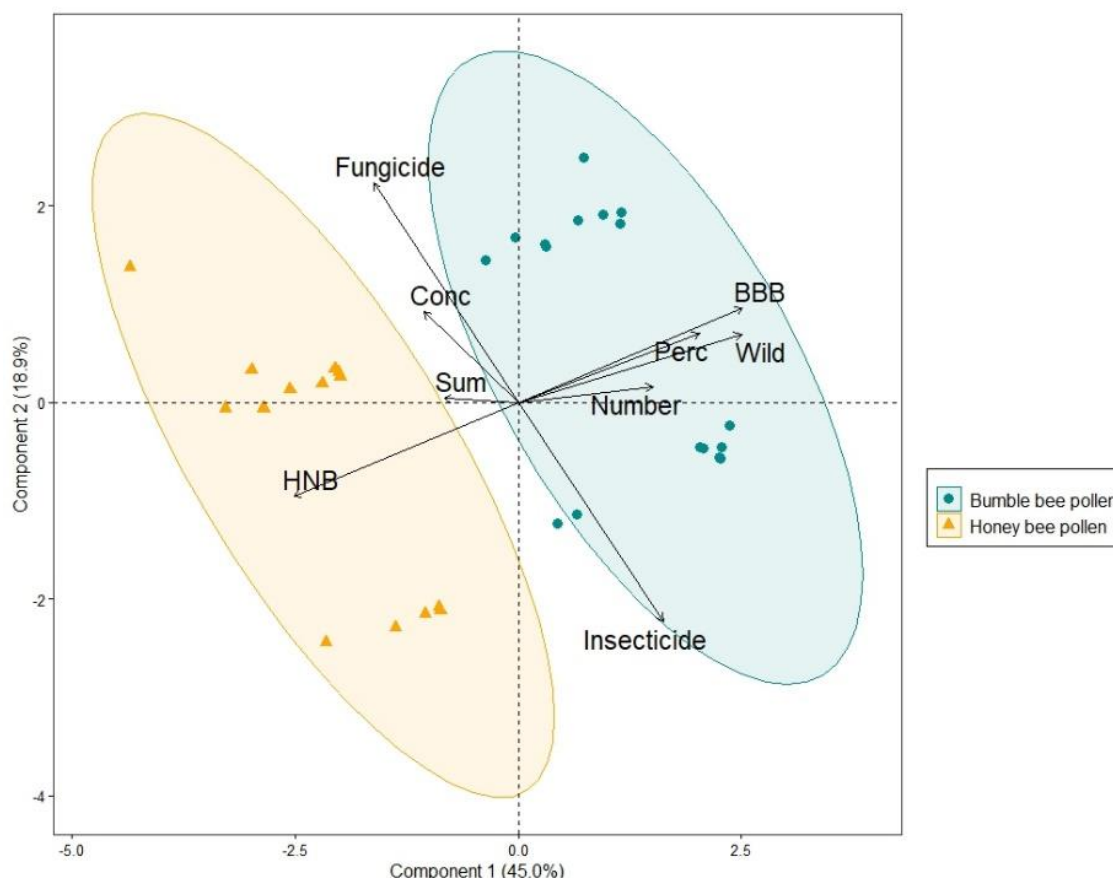


Figure 4.7. Principal Component Analysis of pesticide residues in honey bee (HNB) and bumble bee (BBB) corbicular pollen. The pesticide concentration (Conc), the total concentration (Sum), the total number of active substances per sample (Number), are represented along the active substance type (fungicide or insecticide), the number of wild plants present in the pollen (Wild), the percentage of wild plants present in the pollen (Perc) for each bee species.

4.4.5. Risk Quotient assessment

Using the maximum residues detected in the various pollen types to calculate the acute, and the mean residue levels for calculating the chronic Risk Quotient for adult honey bees (*Apis mellifera*) (Table 4.2), imidacloprid was detected above the respective levels of concern for toxicity. The average acute and chronic RQ values of the residues detected in honey bee (0.002 and 0.006) and bumble bee (0.005 and 0.362) collected pollen, were higher than those detected in crop pollen (0.0001 and 0.001) (see Appendix C Figure C9).

Table 4.2. The acute and chronic Risk Quotients of the maximum and mean concentrations of the seven compounds detected in crop, honey bee and bumble bee pollen.

			Pollen residues (µg/kg)						Risk Quotient					
			Maximum			Mean			Flower		Honey bee		Bumble bee	
	Acute oral LD ₅₀ (µg/bee/day)	Chronic NOED or LDD ₅₀ /10 (µg/bee/day)	Flower	Honey bee	Bumble bee	Flower	Honey bee	Bumble bee	Acute	Chronic	Acute	Chronic	Acute	Chronic
Acetamiprid	14.53	2.42	0.00	0.00	15.32	0.00	0.00	7.7	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Azoxystrobin	> 25	-	1.03	0.05	1.99	0.15	0.02	1.1	< 0.0000	-	< 0.0000	-	< 0.0000	-
Boscalid	> 166	> 15.09	10443.9	5593.4	2181.9	1574.0	1209.4	650.7	< 0.0006	< 0.0010	< 0.0003	< 0.0008	< 0.0001	< 0.0004
Clothianidin	0.004	0.0004	0.00	0.00	11.00	0.00	0.00	5.53	0.0000	0.0000	0.0000	0.0000	0.0264	0.1475
Imidacloprid	0.004	> 0.0000282	0.00	0.00	19.20	0.00	0.00	19.20	0.0000	< 0.0000	0.0000	< 0.0000	0.0498	< 6.5362
Thiacloprid	17.32	0.5	0.00	0.00	44.32	0.00	0.00	22.18	0.0000	0.0000	0.0000	0.0000	0.0000	0.0004
Thiamethoxam	0.005	0.0025	0.00	12.55	17.38	0.00	3.15	2.93	0.0000	0.0000	0.0241	0.0123	0.0334	0.0115

4.5. Discussion

This is the first study directly evaluating pesticide compound residues at a given time before or after their application on the crops in a common agricultural environment, and simultaneously comparing pollen from crop flowers and corbiculae pollen from two essential bee taxa, honey bees and bumble bees, actively foraging on the crops at the same time of the flower collection.

4.5.1. Origin and nature of compound detections

Of the ten pesticide compounds evaluated, seven were detected. Most detections in the samples originated from fields where there was no report of the respective active substance application. Specifically, the compounds azoxystrobin and boscalid were almost constantly present in the collected samples, even though they were only applied in one and three of the 12 sites respectively. It was observed that concentrations of boscalid increased when sampling was performed one day after its application on the crops. The neonicotinoid compounds were only detected in bee collected pollen with no recorded application on the fields. Unlike these seemingly persistent compounds, the herbicide glyphosate, and the fungicide prothioconazole were not detected. This can be attributed to the fact that the application of these compounds on the fields took place several days (e.g., prothioconazole) up to several months before our sampling (e.g., glyphosate), hence their residues in pollen have dissipated enough to be below our method's LOD. Overall, the most common pesticide combination detected was that of azoxystrobin, boscalid and thiamethoxam.

These are in accordance with our research that took place in 2019, where we evaluated the same pesticide compounds for residues in pollen and nectar of OSR crops and *Rubus fruticosus* agg. wildflowers (Zioga et al., 2023; Chapter 3), and we found that: six out of the 11 evaluated compounds were detected, some pesticide compounds were less persistent than others both in terms of their final occurrence and their level of residues in the samples, constituting the time between the product application and sampling crucial for the result; azoxystrobin and boscalid were the common contamination denominators and along with clothianidin, another neonicotinoid compound, they formed the most frequently detected pesticide mixture. The only exception was the pattern of the neonicotinoid detections in the samples; all five were only detected in bee collected pollen, and not in crop pollen, in the study of 2020, while in 2019 clothianidin and thiacloprid were found in both plant species analysed. The non-detection of neonicotinoids in 2020 crop pollen might show that neonicotinoids have reached very low levels in soil, thus any translocated residues could not be detected with our method. Moreover, considering that in 2019 our results revealed detections mainly in nectar samples at <LOQ levels

and the (albeit slow) degradation of these compounds in the environment, the likelihood of detecting neonicotinoid residues in crop pollen in 2020, were much lower.

More compounds and higher concentrations were detected in samples collected from OSR, although more pesticide compounds were applied on BEA crops. This could be mainly attributed to the fact that more of the evaluated compounds in the present study were applied in OSR crops. Furthermore, these two crops belong to different plant families and their plant physiology and flower morphology varies, so might their pesticide residues in pollen (Gierer et al., 2019). For instance, when azoxystrobin was applied on a BEA crop field and sampling was performed within one to seven days after, no residues were detected in any of the pollen types. This was not the case in 2019, where azoxystrobin residues were detected several days after its application in OSR crops (Zioga et al., 2023; Chapter 3). Additionally, when OSR and BEA flowers co-occurred on a field and samples were taken from both for all three pollen types, the residues of boscalid sprayed the previous day on the crop were higher in OSR than BEA pollens. Even though these results come from a single field, they may imply that due to flower morphology or other plant characteristics, pesticide residues of the same pesticide product, applied the same way, may indeed slightly differ for each crop. In the case of a Fabaceae plant (e.g., BEA crop) residues may be lower since the anthers are covered by the flower's petals, and thus, protected from the direct pesticide product application, while in a plant belonging to Brassicaceae family (e.g., OSR crop), the anthers are directly exposed during the pesticide application (Gierer et al., 2019).

4.5.2. General patterns of specific compound detections

Overall, 66 studies performed worldwide have evaluated pesticide residues in plant and bee collected pollen (see Appendix C Figure C1), but only three of them have compared pollens of different bee species (David et al., 2016; Graham et al., 2021; Rolke et al., 2016), and only two have compared bee collected pollen with plant pollen (Botías et al., 2015; David et al., 2016). These studies performed their sample collection independently of the pesticide applications and pollen from crop flowers, and fresh honey bee and bumble bee pollen were not simultaneously collected. Glyphosate has been previously detected in plant and bumble bee corbicular pollen (Thompson et al. 2022; Zioga et al., 2022; Chapter 5), and prothioconazole and its main metabolite prothioconazole-desthio were detected in honey bee corbicular pollen (Böhme et al., 2018; Prado et al., 2019; Raimets et al., 2020). The reason these compounds were not detected in our study is mainly attributed to the fact that these compounds were applied on the fields several days to several months before the crop flowering period and consequently, our

sampling time. Although this is the first study providing pesticide residue data in the three pollen types simultaneously, the four neonicotinoids (clothianidin, imidacloprid, thiacloprid and thiamethoxam) and two fungicides (azoxystrobin and boscalid) that we detected in the evaluated pollen types in our study had already been detected in similar pollen types in other studies (see Appendix C Figure C1). Additionally in our study, however, we recorded acetamiprid residues in bumble bee corbicular pollen for the first time.

4.5.2.1. Pesticide detections across the different pollen types

Our data revealed that only the two fungicides azoxystrobin and boscalid were common across all three pollen types, and along with the neonicotinoid thiamethoxam, they were common in both bee species corbicular pollen. Moreover, our findings demonstrated a higher number of different pesticide compounds detected in bee collected pollen and specifically bumble bee pollen, followed by crop pollen, and lastly honey bee collected pollen. According to the literature (see Appendix C Figure C1), more detections were observed in honey bee pollen, then crop pollen and few detections in bumble bee pollen, but this relationship seems to be related to the number of studies performed for each pollen type (respective number of studies = 49 > 14 > 6). In the study of David et al. (2016), where plant, honey bee and bumble bee pollens were co-evaluated, the compounds boscalid, thiacloprid and thiamethoxam occurred in all three matrices, but plant pollen had the highest number of different compound detections, followed by honey bee pollen. However, in that study bumble bee pollen originated from stored pollen inside the bumble bee hive, collection of pollen samples was performed in different time intervals, and bumble bee nests were placed in different fields than honey bee hives. In the study of Graham et al., (2021), when honey bee and bumble bee pollens were collected from sprayed fields during the same year, azoxystrobin, boscalid, clothianidin, and imidacloprid were detected in both pollen types, while acetamiprid and thiamethoxam were only found in honey bee pollen. The main differences with our study that could help account for this discrepancy, are that we used different focal crops (hence different farming practices, different visitation rates for the two bee species etc.), and that neonicotinoid compounds were not applied in any of our fields.

We detected higher mean concentration levels in crop pollen, followed by honey bee and then bumble bee corbicular pollen. In contrast, based on literature worldwide analyses, the lowest mean levels were recorded in crops, followed by honey bee and the highest concentration levels were in bumble bee pollen. The high mean levels of pesticides detected in our crop pollen samples and the worldwide analysis of bumble bee pollen samples are related to the

concentrations of the compound boscalid, which is also the compound that had the highest residues in honey bee pollens, both in our study and worldwide (see Appendix C Table C7).

4.5.2.2. Fungicide, herbicide, and insecticide detections

In our research we recorded more fungicide detections followed by insecticides, while no herbicides were detected, albeit the highest number of our evaluated compound detections from worldwide studies is largely attributed to insecticides, with fewer than half of this number of detections reported for fungicides, and very few detections observed for herbicides. This difference could result from the fact that pesticide residue studies have mainly focused on certain compounds, especially the neonicotinoid insecticides, neglecting the numerous other active substances applied on crops in modern agriculture (Cullen et al., 2019; Zioga et al., 2020; Chapter 2), potentially presenting a different reality of what is contaminating pollen and likely underestimating the levels of pollen contamination on a real agricultural field environment (Toselli and Sgolastra, 2020). As reported previously in the literature, insecticide detections exceeded those of fungicides across the various pollen types (e.g., plant pollen, honey bee and bumble bee corbicular pollen), and only in solitary bee pollen was an equal number of fungicide and insecticide detections observed (Rolke et al., 2016; Schwarz et al., 2022; Šlachta et al., 2020). Our results indicated that fungicides, but not insecticides, were detected in crop pollen; more fungicides and few insecticides were detected in honey bee corbicular pollen; while more insecticide than fungicide detections were observed in bumble bee pollen. The number of fungicides evaluated in our study was lower than that of insecticides ($2 < 5$), but the number of recent fungicide applications compared to no application of neonicotinoid insecticides, could justify this occurrence pattern in our samples.

In our study, the average level of fungicide concentrations was slightly lower than the average fungicide concentrations reported by the literature. According to our findings, fungicide levels were higher in crop pollen, followed by honey bee pollen and lower in bumble bee pollen, but according to the literature, bumble bee pollen had the highest average fungicide concentration (David et al., 2016; Graham et al., 2021; Kuivila et al., 2021). The low average number of fungicides in crop pollen from other studies, is a result of random sampling (i.e., not related to the pesticide applications) and to the fact that non-crop species were also analysed for pollen residues (Bloom et al., 2021; David et al., 2015, 2016; Willis Chan et al., 2019). The average concentrations of neonicotinoids detected in our study were higher compared to those reported from the literature (Cutler and Scott-Dupree, 2014; David et al., 2016; Graham et al., 2021; Rolke et al., 2016; Sterk et al., 2016). We found that the concentrations of neonicotinoid residues in

honey bee pollen were lower than those in bumble bee pollen, while according to other studies, honey bees have higher mean of neonicotinoid residues. This could result from the fact that bumble bee species are severely neglected and underrepresented in pesticide residues studies, even during neonicotinoid compound evaluation (Cutler and Scott-Dupree, 2014; David et al., 2016; Graham et al., 2021; Rolke et al., 2016; Sterk et al., 2016). If we consider only the three studies where honey bee and bumble bee pollen is co-evaluated (David et al., 2016; Graham et al., 2021; Rolke et al., 2016), the average neonicotinoid residues in honey bee pollen are lower than those in bumble bee pollen, which is in accordance with our results.

4.5.2.3. Routes of bee exposure to pesticides

Despite not being applied on the sampled fields in our study, neonicotinoid compounds were detected exclusively in bee collected pollen. It has been previously reported that compounds absent from crop pollen may be detected in wildflower pollen of plants growing in the margins of the crop or bee collected pollen (Botías et al., 2015; David et al., 2016; Zioga et al., 2023; Chapter 3), and that in many cases, active substances may be detected in bee pollen despite not having been applied on the fields, indicating that contamination may come from other pollen sources than the focal crops (Favaro et al., 2019; Graham et al., 2021; McArt et al., 2017a; Simon-Delso et al., 2017). Honey bees and bumble bees are regular visitors to OSR and BEA flowers (Cresswell and Osborne, 2004; Nayak et al., 2015). Our bee corbicular pollen analysis demonstrated high percentages of crop pollen in the samples, and this is in accordance with previous findings where on average OSR pollen made up 91% of bee pollen loads (Stanley et al., 2013). This was mainly the case for honey bees, who can carry high proportions of oilseed rape pollen (although they also carry small amounts from other species) (Stanley and Stout, 2014). However, and despite their differences in floral preferences, bee species forage simultaneously on a broad range of wildflowers found growing in field margins (Stanley and Stout, 2014; Wood et al., 2015a). In a previous study, it was indicated that flowering plants other than crops may contribute to bumble bee exposure to pesticides (Graham et al., 2021). Our findings indicate that bees may be exposed to higher fungicide concentrations when foraging on crops and lower when foraging in wildflowers, but may encounter greater number of pesticide residues, especially neonicotinoids, when foraging in wildflowers growing in the margins of the crops (Botías et al., 2015; David et al., 2016).

4.5.2.4. Honey bees and bumble bees may be exposed to pesticides differently

It was found that pesticides are present in floral resources that are visited by honey bees and bumble bees, providing a potential exposure route in an agricultural context. However, the

extent of this exposure may vary among bee taxa, and indeed our results demonstrated a positive relationship between the concentrations of neonicotinoids and the number of wild plants present in bumble bee pollen, but the same was not verified for honey bees. Moreover, a greater number of compounds were detected in bumble bee pollen. Indeed, all five neonicotinoids plus the two fungicides were detected in bumble bee pollen, but out of all the neonicotinoids, only thiamethoxam was found in few honey bee pollen samples. Honey bee exposure to pesticides through pollen has previously been reported to differ from bumble bee exposure in various crops (David et al., 2016; Graham et al., 2021), where a greater number of pesticides were detected in honey bee pollen likely reflecting their larger foraging range (Gradish et al., 2019). However, in these studies the timing of sample collection was not standardised in relation to pesticide application (David et al., 2016; Graham et al., 2021), and sample collection was performed differently for the two bee species (David et al., 2016). Varying foraging behaviours, flight ranges, and flower handling altogether, could affect how much pesticide is collected by foraging bees (Gradish et al., 2019). It should be further investigated whether the various bees taxa are exposed differently to fungicides and insecticides and, if this is the case, elucidate why.

4.5.2.5. The case of the neonicotinoid insecticide thiamethoxam

Rundlöf et al. (2015) found that bumble bee colonies were negatively influenced by clothianidin treated OSR, which is the major bioactive metabolite of thiamethoxam, but that honey bees showed no significant impairment. Thiamethoxam has been previously detected in honey bee (Botías et al., 2015; David et al., 2016; Graham et al., 2021) and bumble bee pollen (David et al., 2016), even in cases when it was not applied on the focal crops (Graham et al., 2021). It was among the most frequently detected compounds in bumble bee pollen in a rural environment (David et al., 2016) and the same applied for our bumble bee pollen samples, but at a lower mean concentration compared to that reported in the literature (Cutler and Scott-Dupree, 2014; David et al., 2016). It was also the only neonicotinoid insecticide detected in our honey bee pollen samples at a lower average concentration compared to that of other studies (see Appendix C Figure C1). According to the literature, across the three pollen types, higher thiamethoxam levels have been previously observed in bumble bee pollen and the highest concentration of thiamethoxam was reported in wildflower pollen (David et al., 2016). Nevertheless, Botías et al. (2015) showed that total neonicotinoid concentrations in wildflower pollen were higher than those of OSR pollen, a fact that was mainly attributed to the higher thiamethoxam levels. Based on our findings and according to literature reports, it is suggested

that thiamethoxam contamination is more likely to originate from wildflowers in the margins of the crops, rather than the actual crops.

In the present study, thiamethoxam was detected several years after its last reported application, and it co-occurred in several fields with the fungicides azoxystrobin and boscalid. This co-detection was previously reported in bee related matrices (Mullin et al., 2010; Murcia-Morales et al., 2022; Rondeau et al., 2022; Stoner and Eitzer, 2013; Tosi et al., 2018). Thiamethoxam was detected several years after its last reported application, and it co-occurred in several fields with the fungicides azoxystrobin and boscalid. This co-detection was previously reported in bee related matrices (Mullin et al., 2010; Murcia-Morales et al., 2022; Rondeau et al., 2022; Stoner and Eitzer, 2013; Tosi et al., 2018). Boscalid, which was detected in some instances here in the same samples contaminated with thiamethoxam, has been shown to interact with neonicotinoid insecticides such as thiamethoxam, nearly doubling its toxicity to honey bees (Tsvetkov et al., 2017), even at low doses (Peghaire et al., 2020). It is critical therefore to analyse not only for thiamethoxam residues, but to also investigate if other compounds, that can potentially interact synergistically with thiamethoxam, are also present.

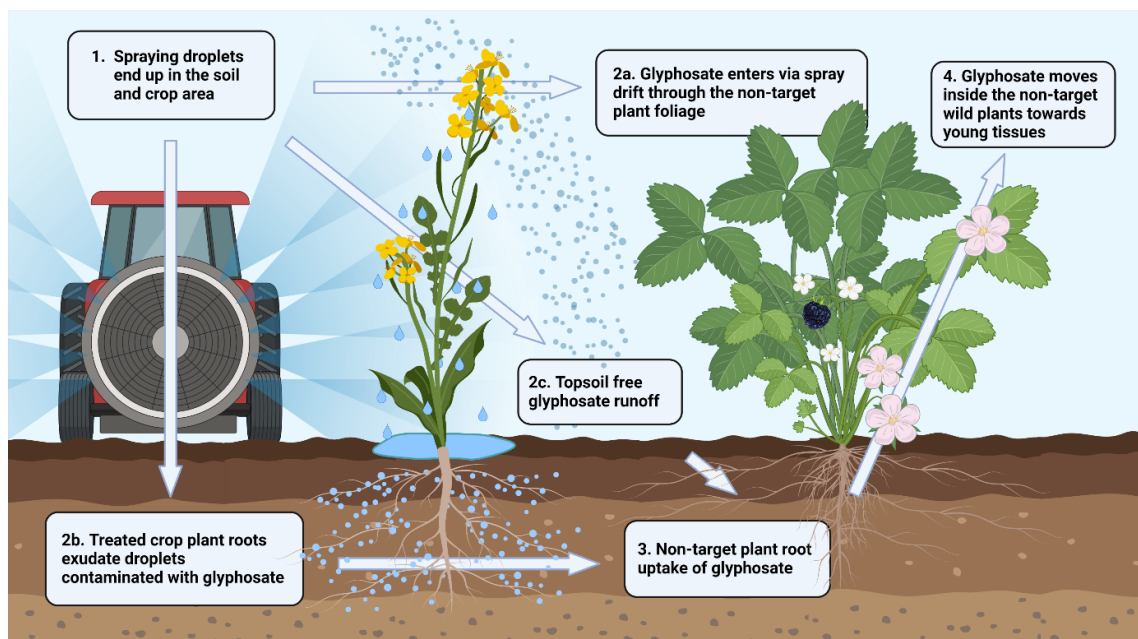
Thiamethoxam alone was found to be highly toxic to bumble bees, compared to other insecticide compounds (Mundy-Heisz et al., 2022). At lower concentrations compared to the average concentrations detected in our study, thiamethoxam exposure alone can impact bumble bee colony initiation success (Baron et al., 2017a), can alter bumble bee food consumption (Kessler et al., 2015; Thompson et al., 2014), may impact bumble bee learning and memory (Stanley et al., 2015b), and can alter bumble bee foraging behaviour and homing success (Stanley et al., 2016) affecting their survival and the delivery of pollination services (Stanley et al., 2016). At higher concentrations to our detected mean, but within the range of the concentrations we detected, thiamethoxam can alter homing success of honey bees (Fourrier, 2020), may adversely affect egg development and feeding rates in bumble bee queens (Baron et al., 2017b), was found to produce a dose-dependent reduction in bumble bee longevity (Mulvey and Cresswell, 2020), may interfere with bumble bee interactions with wild plants (Stanley and Raine, 2016) and impede their ability to provide pollination services (Stanley et al., 2015a). Therefore, in contrast to a recent study (Thompson et al., 2022), our results indicate that thiamethoxam residues in pollen several years after the neonicotinoid ban in EU, may cause sub-lethal effects to bumble bees.

4.6. Conclusion

Pollen from crop flowers, as well as from honey bee and bumble bee pollen loads, was simultaneously sampled from the same locations to compare the quantity and quality of their pesticide content. The average concentrations of pesticide residues in crop pollen were much higher than those of honey bees and bumble bees, but no statistically significant differences were observed among the overall residues. Some compounds were found to leave no residues in pollen after several days after their application (e.g., prothioconazole and glyphosate); however, this is not the case for azoxystrobin, boscalid and the neonicotinoid compounds that seem to be more persistent. Fungicides were mainly detected in crop and honey bee collected pollen while insecticides were mainly detected in bumble bee pollen. Moreover, the number of individual compound detections in bumble bee pollen increased when the number and percentage of wild plants in their pollen increased. Given that no neonicotinoid residues were detected in crop pollen, these findings imply that bumble bee pollen contamination with neonicotinoids come from soil residues, or the wild plants growing near the crops, rather than the actual crops. The same patterns were not observed for honey bees, enhancing the argument that different bee species may be exposed to different compounds, and supporting the argument that more species should be included in pesticide risk assessment for bees. The concentration of imidacloprid in bumble bee collected pollen exceeded the RQ threshold for chronic toxicity, highlighting the high risk from long-term exposure to neonicotinoid compounds. Multiple compounds mixes were observed on a field scale with the fungicides azoxystrobin and boscalid being the common denominators and the neonicotinoid insecticide thiamethoxam being present in most co-occurrence scenarios. This pesticide mix is very relevant for bumble bee pesticide exposure and should be further evaluated for its toxicity.

Chapter 5

Glyphosate used as desiccant contaminates plant pollen and nectar of non-target plant species



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5. Glyphosate used as desiccant contaminates plant pollen and nectar of non-target plant species

5.1. Abstract

Pesticide products containing glyphosate as a systemic active substance are some of the most extensively used herbicides worldwide. After spraying, residues have been found in nectar and pollen collected by bees foraging on treated plants. This dietary exposure to glyphosate could pose a hazard for flower-visiting animals including bees, and for the delivery of pollination services. Here, we evaluated whether glyphosate contaminates nectar and pollen of targeted crops and non-target wild plants. Oilseed rape was selected as focal crop species, and *Rubus fruticosus* growing in the hedgerows surrounding the crop was chosen as non-target plant species. Seven fields of oilseed rape, where a glyphosate-based product was applied, were chosen in east and southeast Ireland, and pollen and nectar were extracted from flowers sampled from the field at various intervals following glyphosate application. Pollen loads were taken from honey bees and bumble bees foraging on the crop at the same time. Glyphosate and aminomethylphosphonic acid (AMPA) residues were extracted using acidified methanol and their concentrations in the samples were determined by a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method. Glyphosate was detected in *R. fruticosus* nectar and pollen samples that were taken within a timeframe of two to seven days after the application on the crop as a desiccant. No glyphosate was detected when the application took place before or more than two months prior to our sampling in any of the evaluated matrices. The metabolite AMPA was not detected in any samples. To gain further insight into the potential extent of translocation within both plants and soil when a crop is desiccated using glyphosate before harvesting, and the potential impacts on bees, we recommend a longitudinal study of the presence and fate of glyphosate in non-target flowering plants growing nearby crop fields, over a period of several days after glyphosate application.

5.2. Introduction

Glyphosate (*N*-(phosphonomethyl) glycine) is a systemic herbicide that has become the most commonly used herbicide worldwide since its commercial introduction in 1974. This is due to several properties: it is non-selective, broad spectrum, phloem-mobile, and available at low production cost (Benbrook, 2016; Duke and Powles, 2008; Ledoux et al., 2020). In Ireland,

glyphosate is the most used active substance in arable crops (DAFM, 2016), and it is found in over 100 products approved for use (DAFM, 2020). Glyphosate is applied to agricultural fields, typically either before crop planting or after harvest, to control the growth of annual and perennial weeds (Silva et al., 2018). It is also used to a lesser extent as a desiccant; a pre-harvest treatment to facilitate the harvesting process by regulating plant growth and ripening (Grao et al., 2022; Karise et al., 2017; Van Bruggen et al., 2018; Zhang et al., 2017). Glyphosate kills plants by inhibiting one part of the shikimate pathway, the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an essential enzyme found in plants, fungi, and some bacteria (Duke and Powles, 2008). The major metabolite of glyphosate is aminomethylphosphonic acid (AMPA) (Blot et al., 2019; Kanissery et al., 2019), and can be found both in plants and in the soil (Arregui et al., 2004; Reddy et al., 2008). Glyphosate is highly soluble and considered non-persistent, with DT_{50} values (time required for 50% dissipation of the initial concentration) of 1.1-13.7 days reported in field studies (PPDB, 2022b). This is in sharp contrast to AMPA, which is classed as persistent with field study DT_{50} values of 283.6 - 633.1 days reported (PPDB, 2022b). Considering the higher persistence of AMPA, and that it has similar toxicological significance to glyphosate (Gomes et al., 2014; Kwiatkowska et al., 2014; Woźniak et al., 2018), both compounds are considered in residue analyses and regulations (Codex Alimentarius, 2013; EPA, 2020).

In many studies, glyphosate and AMPA have been detected in all treated plant products, but also in non-target plants, the soil, animals that feed on crop products, surface and groundwaters (and the organisms that live there), the atmosphere, and humans (Benbrook, 2016; Perez et al., 2011; Van Bruggen et al., 2018). The use of glyphosate as a herbicide that is non-toxic to non-target organisms (e.g., beneficial insects, aquatic life, humans etc) and as a very efficient way for weed control is controversial, and due to its extensive use (Van Bruggen et al., 2018), there is increasing evidence for its ecotoxicological effects on non-target agroecosystem biodiversity (Cuhra et al., 2016; Kanissery et al., 2019; Yamada et al., 2009). Herbicides can impact not only their application area, but also the non-target plant species growing on the margins of that area (Russo et al., 2020). Non-target plants growing in the margins of the main crop are significant in that they provide habitat and food for pollinators in agricultural systems, including both managed honey bees (Pettis et al., 2013) and a range of wild pollinating insects (Grab et al., 2019; Park et al., 2015; Russo et al., 2016). Bees play an important role in both crop and wild plant pollination (Potts et al., 2016), but they are under threat, by the intensification of agriculture and the widespread use of various pesticide products (e.g., insecticides, fungicides, and herbicides) (McArt et al., 2017b; Rundlöf et al., 2015; Woodcock et al., 2016).

Glyphosate threatens bees indirectly by reducing the availability of nutritional resources (Bohan et al., 2005; Gill et al., 2018; Sharma et al., 2018). Even though there is conflicting evidence for glyphosate's toxicity to bees, the recent meta-analytical review on papers that evaluated the effect of glyphosate on bee mortality concluded that glyphosate can be toxic to bees and highlighted the need for further evaluation of both lethal and sublethal effects in bee species other than honey bees (including stingless and solitary bees) (Battisti et al., 2021). AMPA concentrations found in beebread led to sublethal exposure to bees (El Agrebi et al., 2020), but the number of studies is still too low to draw any definitive conclusion on the effects of AMPA on bees (Battisti et al., 2021).

Glyphosate has been detected in various hive matrices, including pollen, nectar, honey, and honey bee larvae (Berg et al., 2018; Chamkasem and Vargo, 2017; Ferreira de Souza et al., 2020; Karise et al., 2017; Kyriakopoulou et al. 2017; Rubio et al., 2014; Thompson et al., 2014b, 2019; Zhu et al., 2017a; Zoller et al., 2018), and sometimes in remarkably high concentrations (Thompson et al., 2014b; Kyriakopoulou et al., 2017). However, there is still a lack of information on the field realistic concentrations of glyphosate in plant derived pollen and nectar, and further data collection is required from a regulatory perspective for that specific compound (Kyriakopoulou et al., 2017). A recent review has demonstrated that systemic insecticides and fungicides may contaminate plant pollen and nectar of both target and non-target plant species, and the non-target exposure route to pesticides has been identified as more relevant for all bee species and not just honey bees (Zioga et al., 2020; Chapter 2). Nevertheless, an accurate and complete understanding of the presence, persistence, and translocation of glyphosate residues in crop and non-crop plant pollen and nectar is still lacking (Zioga et al., 2020; Chapter 2).

Hence, in the present study, we provide more information on the concentration of glyphosate in plant derived pollen and nectar and examine whether this contamination affects crop and non-target plant species. For this, we selected two plant species (a crop and a non-target wild plant) suggested by previous studies as good candidates for residue evaluation studies (Zioga et al., 2020; Chapter 2): the oilseed rape crop species (OSR - *Brassica napus* L.) and the non-target wild plant blackberry (BAB - *Rubus fruticosus* L. agg.) that grows on the margins of the crop field and is a highly valuable food resource for flower-visiting insects (Wignall et al., 2020). Specifically, we tested whether glyphosate residues could be detected and quantified in 1. Crop nectar and pollen, 2. Wild plant nectar and pollen and 3. Bee-collected pollen.

5.3. Materials and methods

5.3.1. Field locations and sampling method

Seven fields growing winter-planted OSR (WOR, fields 1, 2, 3, and 4) and spring-planted OSR (SOR, fields 5, 6, and 7) were selected in the East and Southeast of Ireland (Figure 5.1) and sampled during crop flowering (March/April for WOR, June/July for SOR) in 2019 and 2020.

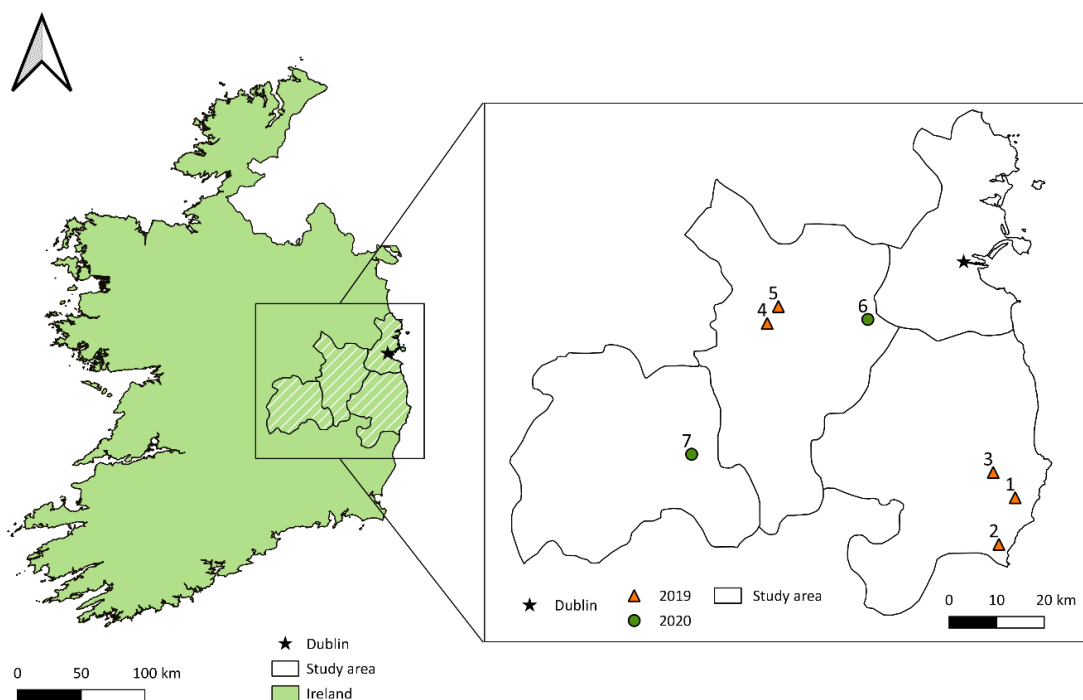


Figure 5.1. The map of the seven sampled oilseed rape fields in 2019 and 2020.

The choice of fields was based on whether the active substance, glyphosate, had been applied on the crops at any stage of the cultivation period. For each field, all information related to glyphosate applications during the crop season (e.g., product applied, date and rate of application etc.) (see Appendix D Table D1) along with general information regarding the field (e.g., crop variety and cropping history) was recorded after interviewing the farmers (see Appendix D Table D2). In 2019, WOR and SOR flowers were collected for pollen and nectar extraction. That year, blackberry (BAB) flowers from plants growing on the edges of the fields where OSR crops were cultivated were also collected for pollen and nectar extraction. In 2020, SOR flowers were collected for pollen extraction. In fields 2 and 4, BAB flowers were collected twice 2-7 days after the glyphosate application on the crop.

To obtain sufficient nectar and pollen for chemical analysis (100 mg and 100 µL nectar), a minimum of 1000 flowers from each plant species in each field during each sampling event were collected. Nectar was collected into individual eppendorf tubes for each sample with a microcapillary tube and pollen was retrieved from the anthers, after incubation in 37 °C for 24 hours (e.g., see Botías et al., 2015). In 2020, honey bee and bumble bee workers foraging for pollen on the crop were also collected (see Appendix D Table D2). To acquire sufficient pollen for chemical analysis, a minimum of 20 honey bees and 15 bumble bees observed actively foraging on the crops, and carrying relatively large amounts of corbicular pollen, were haphazardly collected from each field. The bees were caught by net and placed in plastic vials in coolers before being transferred to the lab. All samples were weighed and stored at -25 °C prior to analysis. Pollen pellets collected from each individual bee species collected in every site were combined and palynological analysis was performed. 500 grains of pollen were counted for each slide and identified based on their morphology, using a standard key (Crompton and Wojtas, 1993), pollen reference samples collected on the sampling fields, and online pollen reference collections (e.g., Chadwick et al., 2019).

5.3.2. Pesticide residue analysis

5.3.2.1. Chemicals and reagents

Certified analyte standards of glyphosate and AMPA, formic acid, ammonium bicarbonate ($\text{H}_2\text{N}_2\text{CO}_3$), Millipore Millex syringe filters with hydrophobic PTFE membrane (pore size 0.22 µm and 20 mm diameter) and low-adsorption LC-MS certified vials were obtained from Sigma Aldrich, Ireland. The internal standards (IS) Glyphosate-2- ^{13}C , ^{15}N and AMPA- ^{13}C , ^{15}N were purchased from LGC, Ireland. The HILICpak VT-50 2D column 2.1 × 100 mm and 5 µm particle size using HILICpak VT-50G 2A 2 × 10 mm were all purchased from Shodex, United States. All pesticide standards were >98% compound purity and deuterated standards >99% isotopic purity. Ultrapure water was generated using ELGA Purelab Ultra SC MK2 (ELGA, UK), and LC-MS grade acetonitrile (ACN) and methanol (MeOH) were obtained from Sigma-Aldrich, Ireland. Individual standard pesticide (native and deuterated) stock solutions (1 mg/mL) were prepared in ultrapure water. An additional IS mixture of the deuterated pesticides at 1000 ng/mL was also prepared. Calibration solutions were freshly prepared from the stock solutions. All stock solutions were preserved at -25 °C in dark conditions.

5.3.2.2. Nectar extraction

100 µL of nectar was placed into a 15 mL PTFE centrifuge tube. A solution of H_2O /ACN (70:30) was added so that the volume of the final extract was 1 mL. The nectar sample was then

homogenized using a vortex and centrifuged at 2500 rpm for 5 min. The solution was filtered through 20 mm PTFE hydrophilic syringe filters (Sigma-Aldrich) into an LC-MS vial.

5.3.2.3. Pollen extraction

A modified protocol of the Quick Method for the Analysis of numerous Highly Polar Pesticides in Foods of Plant Origin via LC-MS/MS (QuPPE-Method) was used for pollen, as suggested by the (European commission, 2021). Briefly, 100 mg of pollen was weighed in a 50 mL PTFE centrifuge tube to which 5 mL of acidified MeOH (1% v/v formic acid) was added. The tube was vortexed for 1 min. The tube was centrifuged at 4500 rpm for 10 min. The supernatant was transferred into a 15 mL PTFE centrifuge tube and was placed in -25 °C for 1 hour. An aliquot of the supernatant was mixed with H₂O/ACN (70:30). The solution was filtered through 20 mm PTFE hydrophilic syringe filters (Sigma-Aldrich) into an LC-MS vial.

5.3.2.4. LC-MS/MS analysis

The analysis of the two compounds in both nectar and pollen was performed on an Agilent 1290 Infinity II Multisampler LC System coupled to an Agilent 6470 Triple Quadrupole mass spectrometer (LC-MS/MS). Compound separation was achieved on a HILICpak VT-50 2D column (2.1 × 100 mm and 5 µm particle size, Shodex) using a column oven at 30 °C and a flow rate of 0.1 mL/min. Mobile phase A consisted of 50 mM ammonium bicarbonate in H₂O and mobile phase B comprised of pure ACN. A 70% mobile phase A elution programme was used, and the total time of the LC-MS/MS analysis was 25 min. The injection volume was 20 µL. The ion spray voltage was set at -3000 V for negative ionization. Source temperature was set at 300 °C. Nitrogen was used as a curtain gas (8 L/min), nebulizer gas (30 psi), sheath gas flow (12 L/min), and sheath gas temperature was 350 °C. Agilent Mass Hunter Workstation Data Acquisition Version 10.0 software was used to control LC-MS/MS system and for data acquisition. Quantitative and qualitative analysis was carried out with Agilent Mass Hunter Workstation Qualitative Analysis software version 10.0, based on two most abundant precursor ion and product ion MRM transitions, and their characteristic retention time. The compound-specific LC-MS/MS retention times (Rt), quantifying transition ions (Q) and qualifier transition ions (q) for glyphosate, AMPA and their ISs are shown in Appendix D Table D3.

5.3.2.5. Method validation

Validation experiment was carried out according to requirements of Document No. SANTE/12682/2019 guidance (Appendix D Table D4) (European Commission, 2020a). To prepare the pollen matrix-matched calibration, pollen free from pesticide residues was used as blank.

For the nectar matrix-matched calibration, a nectar surrogate was created by mixing 6 g of glucose and 3 g of fructose in 25 mL of ultrapure water (Martel et al., 2013).

5.3.3 Risk evaluation

For evaluating the potential risk that pesticide residues detected in pollen and nectar pose specifically to honey bees, the BeeREX model was used (Thompson 2021; US EPA 2015), as previously described in (Rondeau and Raine, 2022).

5.4. Results and discussion

Residues of the active substance were detected in BAB pollen and nectar from three WOR fields sampled in 2019 (field 1, 2 and 4) (Table 5.1, Figure 5.2).

Table 5.1. Glyphosate residues detected in oilseed rape crop and wild blackberry (*Rubus fruticosus*) pollen and nectar, and honey bee and bumble bee collected pollen.

Field	Glyphosate residues (µg/kg)				
	Oilseed rape			Blackberry	
	Flower Pollen	Flower Nectar	Bee pollen	Flower Pollen	Flower Nectar
1 ^a	-	<LOD*	-	-	96.8±4.4
2 ^b	-	<LOD*	-	<LOD	135.4±0.4 ¹
			-	-	205.7±6.8 ²
3	<LOD	<LOD	-	<LOD	<LOD
4 ^c	-	<LOD ^{1*}	-	<LOQ	<LOQ ¹
	-	<LOD ^{2*}	-	-	187.3±9.2 ²
5	<LOD	<LOD	-	<LOD	<LOD
6	<LOD	-	<LOD	-	-
7	<LOD	-	<LOD	-	-

^{1,2} The numbers refer to the first and second sampling performed on fields 2 and 4 for the respective samples.

* This symbol refers to samples that were collected before the pesticide application on the crop.

^{a,b,c} The *R. fruticosus* flower sampling of these fields was performed after two (field 1), after three and five (field 2), and after two and seven days (field 4) from the application of glyphosate on the crop.

The dash (-) implies that the respective samples were not evaluated for glyphosate residues.

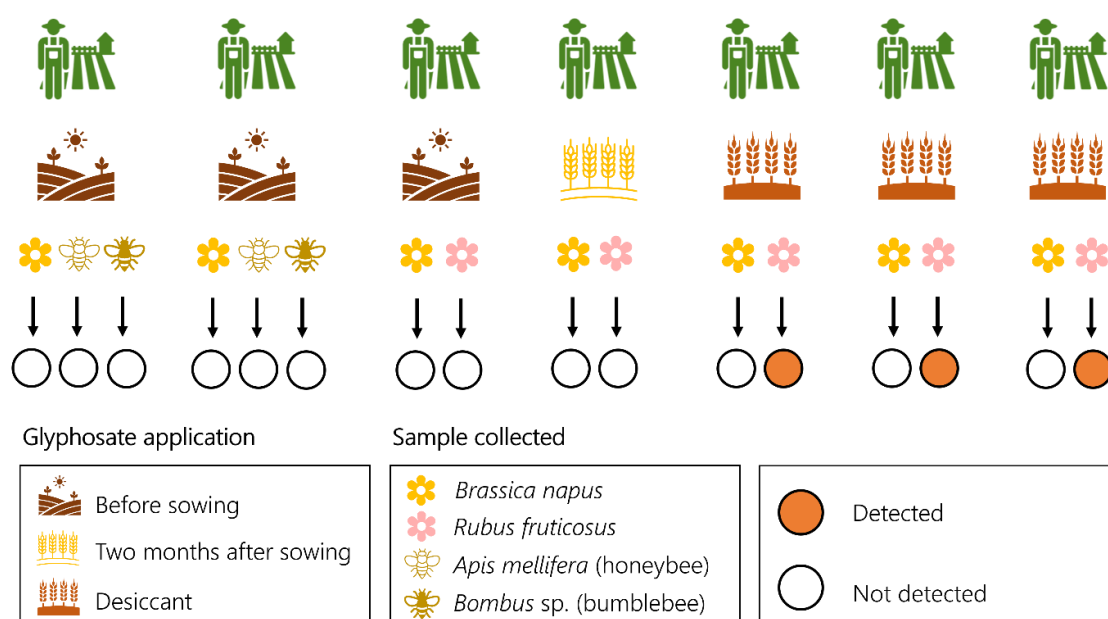


Figure 5.2. Application timing of the glyphosate product in the seven fields and the respective detections in the collected samples.

The detections in BAB pollen and nectar originate from fields where the glyphosate product was applied as a desiccant within one week of sampling. On the other hand, when used as a pre- or post-emergence spray, no residues were detected in nectar and pollen of the crop, or the wild plant (Figure 5.2) sampled more than 2 months after spraying. In crop related matrices, residues increase in grain, fodder, and oil when glyphosate is used as a desiccant before harvest (Cessna et al., 1994, 2000; FAO, 2005; McNaughton et al., 2015; Zhang et al., 2017). Residues of compounds used as desiccants have been previously reported in wild plants (Vahl et al., 1998), in the environment (e.g., water) (Zhao et al., 2013), and even honey bee matrices like honey and comb pollen (Erban et al., 2017), suggesting potential exposure on non-target organisms. Here, we report for the first-time glyphosate residues in nectar and pollen of a non-target wild plant species growing in the margins of the main crop area (0 - 1 m distance), when the herbicide product is applied as desiccant to facilitate the harvesting process. It is suggested that off-target herbicide drift can adversely affect wild plant species on the margins of the crop fields (Gove et al., 2007; Marrs et al., 1993), changing the interactions between non-target plants and flower-visiting insects (Russo et al., 2020). Research has demonstrated that non-target movement of glyphosate during application can be up to 10% of the applied rate (Al-Khatib and Peterson, 1999; Ellis and Griffin, 2002), and as little as 1-5% of the recommended application rate can negatively impact the mutualistic interactions with pollinators (Dupont et al., 2018).

Although through our study it is difficult to determine how glyphosate contamination occurred in pollen and nectar of a non-target wild plant species, it is very important to identify the

potential contamination pathways that may have caused this off-target plant contamination. Generally, glyphosate applied as foliar spray for weed control can potentially reach unintended areas and non-target plant tissues through three main pathways: 1) spray drift, 2) runoff, and 3) root uptake (Kanissery et al., 2019; Laitinen et al., 2007; Marrs et al., 1989; Russo et al., 2020) (Figure 5.3).

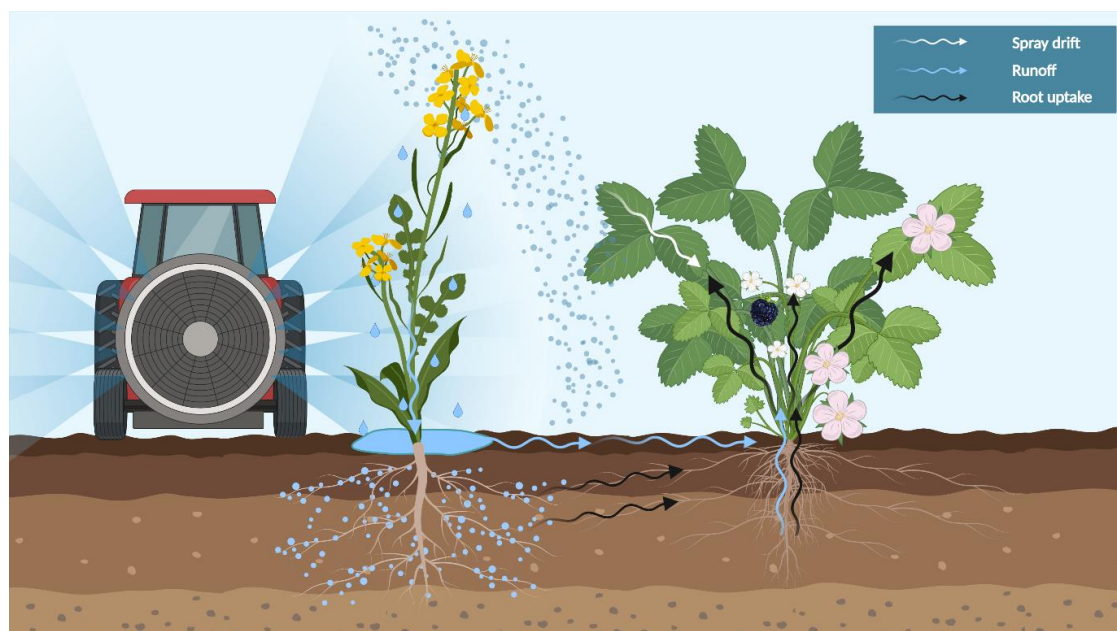


Figure 5.3. Potential glyphosate translocation pathways in soil and target and non-target plant species.

Spray drift is the movement of droplets of pesticide spray away from the target application area, caused by wind (Ochoa and Maestroni, 2018). This contamination pathway is dependent on several factors, some of which include wind intensity and direction, droplet size, temperature, and height of spraying nozzle (Ochoa and Maestroni, 2018). Foliar application of glyphosate contaminated non-target plants with the active substance for at least four weeks after the treatment (Neuman et al., 2006). Glyphosate spray drift may contaminate plant pollen and nectar directly, or by reaching the foliage of non-target plants, where it can be absorbed by the plant leaves other green tissues via diffusion (Duke and Powles, 2008), and be translocated to the entire plant, accumulating preferentially in young growing tissues (Franz et al., 1997). In our study, when glyphosate was applied as desiccant, the farmers advised that product application directions provided by the manufacturer were used, and the suggested measures to avoid spray drift were followed (e.g., zero to mild wind, spray boom as low as possible, no spraying close to the field boundaries, spraying characteristics according to the label recommendations etc.) (HSE, 2022). The absorption and translocation of glyphosate inside the plant may be influenced by the formulation, as those containing surfactants (and adjuvants) have a higher rate of

absorption compared to glyphosate water solutions (Doublet et al., 2009), increasing the uptake and translocation of glyphosate in plants. This should be considered in higher tier risk assessment studies, when decisions should be made about whether the active substance or the commercial formulation should be sprayed on the evaluated plants. In our study, field 4 only, an adjuvant was applied as pod sticker (i.e., a product designed to prevent pod shatter by coating the crop with a thin film of polymer) at the same time as the glyphosate product application.

Although glyphosate is typically sprayed onto plant foliage, some chemical can reach the soil through spray-drift, by being washed off plant surfaces during precipitation (Al-Kathib and Peterson, 1999; Ellis and Griffin, 2002; Kremer and Means, 2009; Saunders and Pezeshki, 2015), by exudation from roots or death and decomposition of treated plant residues (Kanissery et al., 2019; Laitinen et al., 2007; Mamy et al., 2016; Neumann et al., 2006; Tesfamariam et al., 2009; Von Wiren-Lehr et al., 1997). Glyphosate and AMPA display similar behaviour in terms of sorption and degradation (Feng and Thompson, 1990; Simonsen et al., 2008; Bento et al., 2016), as they strongly sorb to soil minerals (Sprankle et al., 1975; Hance, 1976; Roy et al., 1989; Piccolo and Celano, 1994; Borggaard and Gimsing, 2008; Sheals et al., 2002; Gimsing and Borggaard, 2002; Gimsing et al., 2004; Veiga et al., 2001; Vereecken, 2005; Gimsing et al., 2007), accumulating in the top layer of soils (depth of 0-15 cm) (Bento et al., 2019; Kanissery et al., 2019; Silva et al., 2018). In agricultural settings, repeated glyphosate applications increase the risk of glyphosate and AMPA accumulation in topsoil (Benbrook, 2016), considering that glyphosate and AMPA may persist in soil for long periods, under certain conditions (e.g., dry soil, low temperatures, soils with strong adsorption capacity etc.) (Bento et al., 2019; Kanissery et al., 2019). However, absorbed glyphosate and AMPA can be desorbed under certain conditions such as the addition of fertilizers (Borggaard and Gimsing, 2008; Gimsing et al., 2004; Simonsen et al., 2008; Zhou et al., 2010), or heavy rain events shortly after glyphosate application, increasing the risk of glyphosate being transported, deposited, and probably accumulated in nearby areas (Bento et al., 2019; Botta et al., 2009; Candela et al., 2010; Degenhardt et al., 2012; Gjettermann et al., 2009; Hanke et al., 2010; Luijendijk et al., 2003; Peruzzo et al., 2008; Shipitalo et al., 2008; Stone and Wilson, 2006; Styczen et al., 2011; Todorovic et al., 2014; Ulén et al., 2014; Vereecken, 2005; Yang et al., 2015a, b). Glyphosate and AMPA adsorbed to solid particles suspended in water (Rügner et al., 2014; VandeVoort et al., 2013) is the main mode of offsite contamination via runoff (Yang et al., 2015b), causing a high increase to the off-site contamination levels immediately after glyphosate application (Edwards et al., 1980; Saunders and Pezeshki, 2015). In this study, during the *R. fruticosus* flower collection sporadic heavy

rainfalls were observed throughout the sampling period, but no fertilizer was applied between the timing of glyphosate application and the flower sampling.

Glyphosate translocates within plants, accumulates in roots, and is eventually released into the rhizosphere by the root exudates (Coupland and Casely, 1979; Kanissery et al., 2019; Laitinen et al., 2007; Neumann et al., 2006). From there, it may be reabsorbed by the treated crop or nontreated nearby plants through their root system (Kanissery et al., 2019). Once inside the plant, glyphosate may be transported within the plant xylem in the apoplastic pathway or enter the phloem and get transported to metabolic sinks via the symplastic pathway (Franz et al., 1997). For both foliar and root uptake, glyphosate translocation may be basipetal or acropetal (upwards and downwards), moving toward various tissues, such as meristems, leaves flowers, and fruits (Clua et al., 2012; Dewey, 1981; Duke, 1988; Franz et al., 1997; Giesy, 2000; Shaner, 2009; Tong et al., 2017; Wagner et al., 2003; Walker and Oliver, 2008). There is previously reported evidence of non-target plant contamination through the root system within a two-day period and with an increasing rate for a six-day period (Neuman et al., 2006). BAB plants have a perennial rooting system that comprises of a main vertical root which may grow until 4 m (depending on the soil type) and many secondary roots that grow horizontally to the main root for 30 - 60 cm. There are also several thin roots on the secondary roots in all directions (NSW Department of Primary Industries Weed Management Unit, 2009). Given the growing distance of the BAB plants in the present study (0 - 1 m), it is possible that BAB plants started absorbing glyphosate through their rooting system right after the crop spraying. Glyphosate can enter plants through the roots, or shoots emerging from the root or the trunk (Sharma and Singh, 2001). As glyphosate is stable and not immediately metabolized in many plant species, substantial amounts can be extensively translocated to regions of active growth and accumulate, particularly in young tissues (Reddy et al., 2004). Glyphosate reaches any actively growing tissue or organ (Duke and Powles, 2008), so it may reach pollen. However, it has been suggested that lipophilic pesticides tend to accumulate in pollen, as opposed to hydrophilic compounds (Raimets et al, 2020). Glyphosate is a hydrophilic compound, and it is beyond the scope of this work to investigate whether its presence in the pollen samples in this study was as a result of translocation through the plant from the soil or instead arose through a contamination event, e.g., drift. The physicochemical properties and high solubility of glyphosate in water (PPDB, 2022b) enable it to be translocated via the phloem to the same tissues that are metabolic sinks for sucrose (Siehl, 1997). Even though a detailed pathway has not yet been elucidated, nectar, represents secretions of phloem sap for most species (Heil, 2011). Hence, this is a potential pathway of translocated glyphosate to nectar and the increased

concentration of glyphosate residues in BAB nectar could be a result of the compound's high solubility in water in combination with the rainfalls that occurred after the pesticide application and between the two different sampling dates at those fields. The difference in pollen and nectar concentrations or presence/absence could be linked to the different translocation pathways of glyphosate in pollen and nectar. Whether nectaries and anthers are supplied by phloem or xylem seems to play an important role in the final concentrations of residues in those matrices (Cowles and Eitzer, 2017). However, more research is required to elucidate the factors that affect the translocation of a compound inside the plant until it reaches pollen and/or nectar, with emphasis on the compound physicochemical properties (Gierer et al., 2019) and the plant species evaluated, as translocation patterns are plant species dependent (ATSDR, 2020).

No AMPA residues were detected in any of the 25 samples evaluated ($< \text{LOD}$). Also, no residues of glyphosate were detected in any of the OSR pollen and nectar samples of all seven fields, nor in any of the honey bee and bumble bee collected pollen from the two fields sampled in 2020 (field 6 and 7). Most of these samples were collected either prior to glyphosate application (e.g., OSR flowers of fields 1, 2 and 4) or more than two months (> 75 days) after the glyphosate application on the field (e.g., OSR and BAB flowers of fields 3, 5, 6 and 7), and the only exception was the BAB pollen of field 2 where sampling was performed three days after the application, but no residues were detected. Glyphosate and AMPA are both adsorbed to clay and organic matter particles (Banks et al., 2014; Okada et al., 2016; Tang et al., 2019). Once adsorbed their degradation is very slow and both compounds are characterized as persistent in soils (EFSA, 2015d). The rates of glyphosate and AMPA degradation are highly variable, ranging from a few days up to one or two years (Saunders and Pezeshki, 2015; Silva et al., 2018; Yang et al., 2015a), with AMPA being more persistent than glyphosate (Bento et al., 2016). Degradation seems to increase in soils with increasing pH, high percentage in organic matter, higher temperatures, well-drained soil systems (Saunders and Pezeshki, 2015), increasing concentrations of inorganic soil phosphate, and decreasing mineral concentrations (Glass, 1987; Gerritse et al., 1996; Piccolo et al., 1994; Plimmer et al., 2004; Smith and Oehme 1992; Sprankle et al., 1975). Hence, a combination of various field characteristics (e.g., soil properties, plant species etc.) and the impact of external factors (e.g., climate, fertilization management, chemical interventions etc.) may be the reason why no AMPA was detected in OSR pollen and nectar in the present study. Glyphosate dissipation rate in plant matrices is estimated to be within the range of 2.2 to 17.0 days (PPDB, 2022b) and metabolism of glyphosate within the plant occurs slowly (Doublet et al., 2009; Reddy et al., 2004; Smith and Oehme, 1992). Hence, the absence of AMPA in the BAB

pollen and nectar may be attributed to the fact that glyphosate was not yet breaking down to an extent that could be detected with our method.

The fact that glyphosate and AMPA cannot be analyzed in the same multiresidue analysis along with other pesticides due to their chemical characteristics (e.g., high polarity) (Pareja et al., 2019; Vicini et al., 2021), adds an extra cost and complexity to analysis and may account for the absence of this compound in the list of the screened pesticides in most studies evaluating pesticide residues in honey bee related matrices (Toselli and Sgolastra, 2020). However, when it is evaluated, glyphosate is among the pesticides that are frequently detected in beehive matrices (Berg et al., 2018). Residues have been detected in larval honey bees (up to 19.5 mg/kg) (Thompson et al., 2014b), in beebread (47.2 - 58.4 µg/kg) (Bergero et al., 2021; El Agrebi et al., 2020), brood (34 µg/kg), and nurse bees (289 µg/kg) (Raimets et al., 2020). It was found that honey bees and bumble bees can be exposed to high concentrations of glyphosate when foraging (up to 629.0 mg/kg) (Thompson et al., 2014b, 2022), while residues of both glyphosate and AMPA were detected in stingless bees (< 50 µg/kg) (Guimarães-Cestaro et al., 2020). Regarding AMPA, no residues were found in honey and beeswax, but in beebread, the maximum AMPA concentration reached 250 µg/kg (El Agrebi et al., 2020). In our study, we did not detect any AMPA, while the glyphosate concentrations in BAB nectar ranged from <25 µg/kg (< LOQ) to 205.7 µg/kg with an average value of 156.3 µg/kg and in BAB pollen <35 µg/kg (< LOQ). The glyphosate values we detected in BAB nectar are much lower than the values detected in bee collected pollen and nectar from recently treated plants, but they are higher than those detected in beebread.

In honey bee collected pollen and nectar, and bumble bee collected pollen from plants that were directly sprayed with glyphosate, concentrations declined over time after the initial spray application (Thompson et al., 2014b, 2022). On the other hand, the glyphosate residues detected in BAB nectar of fields 2 and 4 where the herbicide was applied as a desiccant to the crop, seem to increase over time. Even though the number of data points is too low to draw definite conclusions, the number of flowers collected per sample in each field (> 1000 flowers) represents a big sample area in each field. Yet, it remains uncertain whether contamination of non-target plant pollen and nectar may lead to higher concentrations of glyphosate after several days of the pesticide application on the crop and this is something that should be further evaluated. To elucidate the contamination pathway, we recommend an experiment specifically designed for this purpose, where the physiology of the non-target plants is also considered (e.g., the duration of the open flower present on the plants while spraying etc.).

The RQ values did not exceed the thresholds set by both US EPA and EFSA for the foragers and in-hive honey bees risk assessment (Table 5.2). However, our knowledge of the effects of glyphosate on honey bees is scarce and there is significant lack of information on how glyphosate affects the roughly 20,000 species (Orr et al., 2020) of wild bees (Belsky and Joshi, 2020; Franklin and Raine, 2019; Seide et al., 2018). Most of the toxicity studies have focused on honey bees (*Apis mellifera*) with only a few testing the impacts on other bee species (Abraham et al., 2018; Seide et al., 2018). Yet, when other bee species are tested, the herbicide glyphosate is found to be highly toxic (Gomes, 2017; Seide et al., 2018) or has negative effects on their physiological behaviour (e.g., wild bee flight ability) (Gomes, 2017).

In recent years, the worldwide intensive use of glyphosate and its accumulation in the environment and edible products has raised major concerns about noxious side effects of glyphosate and AMPA on plant, animal, and human health (Van Bruggen et al., 2018, 2019, 2021). Based on several publications on potential chronic side effects of glyphosate-based products on human health the World Health Organization reclassified the herbicide glyphosate as probably carcinogenic to humans in 2015 (International Agency, 2015). This implies that honey contaminated with glyphosate residues above the established MRLs may pose a hazard for human health (Ledoux et al., 2020). At the same time, the sublethal effects caused by glyphosate on honey bees could result in the reduction of pollination services, which would impact the worldwide food production (Ledoux et al., 2020).

The concentrations of glyphosate and AMPA in plants vary widely, but the maximum residue limits (MRLs) for glyphosate are set to 0.1-40 mg/kg for most plant products aimed for human consumption (Codex Alimentarius, 2013; Cuhra, 2015; EPA, 2020; European Commission, 2020b; FAO, 2005), and at 50 µg/kg for honey (European Commission, 2013b). Glyphosate has been detected in honey worldwide with concentrations reaching up to 300 µg/kg (Berg et al., 2018; Bergero et al., 2021; Chamkasem and Vargo, 2017; Ferreira de Souza et al., 2020; Pareja et al., 2019; Raimets et al., 2020; Rubio et al., 2014; Thompson et al., 2019; Zoller et al., 2018). A European analysis of several honey samples and other apicultural products in 2018 identified glyphosate among the 30 most quantified compounds with residues often exceeding the MRLs for honey (EFSA, 2020). In a recent study, Pareja et al. (2019) found that glyphosate was detected in more than 81% of tested honey samples and 41% were above the European MRL for honey.

Table 5.2. The maximum residues detected in plant pollen and nectar, the honey bee oral acute (LD₅₀s) and chronic (10-day NOED) toxicity data for glyphosate, and the estimated acute and chronic Risk Quotients for foragers and in-hive honey bees.

	Maximum residues (µg/kg)		Mean residues (µg/kg)				Risk Quotient			
							Acute		Chronic	
	Pollen	Nectar	Pollen	Nectar	Oral honey bee LD ₅₀ (µg/bee)	Chronic NOED (µg/bee/day)	Foragers	In-hive	Foragers	In-hive
Glyphosate	17.5	205.7	17.5	127.5	104.0	10.4	0.000578	0.000279	0.003580	0.001733

In our study we found an average glyphosate concentration of 156.3 µg/kg in BAB nectar, a value that also surpasses the European MRL for honey. This value is relevant to honey MRL because inside *Apis* colonies, after honey bees collect pollen and/or nectar from contaminated sources, glyphosate could further concentrate because nectar is evaporated to make honey and incorporated in bee bread (Seeley, 1995). Values of glyphosate in honey samples may vary depending on plant species and environmental conditions (Farina et al., 2019). In honey collected close to OSR crops glyphosate residues were prevailing upon the rest pesticide compound residues (Karise et al., 2017). In our study we detected glyphosate residues only in wild plant pollen and nectar and not in honey bee and bumble bee collected pollen, which based on our palynological analysis it was mainly collected from the crop, but after several months of the pesticide application. Considering that in Europe the application of glyphosate does not take place in flowering OSR crops (since genetically modified crops are not allowed; Van Bruggen et al., 2018), in combination with the results of the present study, it would be worth evaluating whether glyphosate residues found in honey come from the actual crops, or if their presence in honey is a result of non-target contamination of wild plants.

5.5. Conclusions

Our findings suggest that offsite transport of glyphosate used as desiccant, contaminates pollen and nectar of non-target wild plant species. Based on the present knowledge of glyphosate's fate in environmental matrices (e.g., plants and soil), this transport could be attributed to spray-drift, runoff, and/or root uptake, and is dependent on several environmental factors, field related characteristics and agricultural practices. Bee species can be exposed to high glyphosate residues in the environment and honey bees can transfer these residues into their hives, contaminating honey at concentrations exceeding the established European MRLs and posing a hazard to human health. Knowledge of glyphosate toxicity should be expanded to more wild bee species and respective LD₅₀ and NOED values should be established. With our results we identified pollen and nectar of non-target wild plant species as an exposure route of bees to glyphosate, and we raise concerns about whether the high levels of contamination of honey bee products identified in the literature originate from the crops or are a result of non-target wild plant contamination. In advance of, and to inform the upcoming renewal of market authorization for glyphosate in the European Union, we recommend the immediate investigation of glyphosate as desiccant before harvesting crops, to elucidate the fate of glyphosate residues in soil and non-target flowering plants growing near crop fields, over a period of several days after the desiccant spraying.

Chapter 6

General Discussion

6. General discussion

6.1. Summary

The intensification of agriculture has sparked serious concerns about the impacts of current pesticide application practices on pollinator health. Even a rigorous pesticide regulatory system, such as that within the European system, has deficiencies and insufficient risk assessment validation. Holistic risk approaches, as well as close monitoring for pesticide residues in bee related matrices under a realistic agricultural environment and vigilant adherence to protocols, are needed to minimise the adverse effects of pesticide compounds to pollinators and encourage sustainable pesticide use. My PhD thesis focused on three evident yet greatly unexplored aspects: i) the distribution (presence and levels) and persistence of pesticide residues in plant pollen and nectar, ii) the comparison of pesticide distribution between crops and wild plants, and iii) the comparison of pesticide distribution among various pollen types collected by more bee species than just the honey bees.

To address these, I initially conducted a systematic literature review to elucidate the current knowledge on the identity, frequency, and concentration levels of pesticide compounds detected in plant pollen and nectar (Chapter 2). This contributed to identifying major knowledge gaps in terms of the pesticide compound types evaluated to date and their distribution in plant pollen and nectar and helped me select the plant species that were suitable for a field study on bee exposure to pesticide residues. I consequently collected flowers from both crop and non-target wild plant species to determine the levels of potential bee exposure to systemic pesticides representing the three most abundant pesticide categories (herbicides, fungicides, and insecticides) in pollen and nectar matrices in Ireland (Chapter 3). To understand whether different crops represent a distinct hazard for disparate bee species, I compared the residues detected in pollen from two entomophilous crops, and honey bee and bumble bee corbicular pollen simultaneously collected under the same agricultural settings (Chapter 4). Finally, I investigated the distribution of glyphosate and its metabolite AMPA in pollen and nectar directly collected from flowers in an agricultural context and explored the potential contamination pathways of this polar compound (Chapter 5). In the current chapter, I synthesize and discuss the main findings, explore implications, address thesis limitations, and provide recommendations for future work and research.

6.2. Major findings

The main findings of my thesis, answer essential questions of the contamination of nectar and pollen with pesticide residues, are summarized and further discussed below.

Do we have enough information on various pesticide residues detected in pollen and nectar?

The literature review findings (Chapter 2) suggest that there is a paucity of information for most of the world. There is an urgent need to expand the range of compounds evaluated in pesticide residue studies, in different geographical areas, to better understand how the same compounds behave in different climates and landscapes. Although the overall most studied category of pesticides were the neonicotinoid insecticides, the compounds with the highest median concentrations of residues in pollen came from non-neonicotinoid insecticide compounds and fungicides. Herbicide and fungicide residues in pollen and nectar should be evaluated, alongside insecticides. Moreover, co-formulants used in pesticide products along with the main active substances, and certain metabolites that derive from the degradation of the active compounds of those products, should be evaluated too.

Can we define the origin of pesticide contamination and generalize?

As seen in the analysis of samples collected in both 2019 and 2020 (Chapters 3 and 4), most detections were from fields with no recorded application of the respective compounds in that year. Multiple residues of neonicotinoid insecticides were detected in pollen and nectar of all plant species evaluated, as well as bee pollen loads, even though samples were taken several years after, or no reported, neonicotinoid application. Despite a small number of neonicotinoid compound detections in crop pollen and nectar in 2019 (Chapter 3), the 2020 analysis showed a stronger relationship between wild plants and neonicotinoid presence in bee collected pollen (Chapter 4). The fungicides azoxystrobin and boscalid, were the common contamination denominators in all matrices evaluated, despite not having been applied close to the sampling time. Unlike the neonicotinoids, the presence of these fungicides in bee collected pollen seems to be more related to residues in the crops, as they were frequently detected in crop pollen. The compound fluroxypyr applied in rotation crops was not detected in any of the samples and prothioconazole was only detected in samples collected within 16 days of its application. Glyphosate was found to contaminate pollen and nectar of non-target plant species when these are sampled within a week of its application (Chapter 5), yet no glyphosate was detected when the application took place before or more than two months prior to sampling in any of the evaluated matrices. The metabolite AMPA was not detected. The results suggest that the origin

of pesticide residues is compound and time specific, and it is hard to generalize, unless we consider the various pesticide categories separately.

Can we determine the levels of pesticide contamination and generalize?

Generally, higher concentrations were observed in recently treated fields (Chapters 3 and 4). However, that was not the case for neonicotinoid residues, because some of the levels of residues I detected, resembled those from previous studies where neonicotinoids were recently applied (Chapter 4). It is unknown how glyphosate residues behave in nectar and pollen of non-target wildflowers in the long-term, but the glyphosate residues I detected (Chapter 5) were by far the highest pesticide residues detected in pollen and nectar of *R. fruticosus* non-target plants. Again, it seems that pesticide contamination levels in pollen and nectar are compound and time specific.

Which are the most common mixtures detected?

All matrices were contaminated with at least two pesticide compounds, and the most frequently detected compounds were fungicides. I showed that during springtime - a crucial time of the year for most bee species, they can be exposed to high concentrations and a variety of chemical compounds through their food (Chapter 3 and 4). The compounds azoxystrobin, boscalid and thiamethoxam or clothianidin were co-detected on several fields, illustrating that in field conditions bees can be simultaneously exposed to combinations of pesticides from different chemical groups that are known to act synergistically, with negative impact on bee' health.

Is there a difference in the pesticide residues detected in crops and non-target plants?

The results from the literature review showed higher levels of pesticide residues detected in pollen and nectar from crops than wild plants (Chapter 2). From the 2019 sampling, I detected pesticides in pollen and nectar of both *B. napus* and *R. fruticosus* (Chapter 3). Overall, more residues were detected in *B. napus* pollen and nectar than in the wild plant, and *B. napus* pollen had the highest mean concentration of residues. Higher concentrations were observed in the crops for all compounds except for glyphosate and thiacloprid that were only detected in *R. fruticosus* pollen and nectar. With my results I identified pollen and nectar of non-target wild plant species as an exposure route of bees to glyphosate, which raises concerns about whether the high levels of contamination of honey bee products identified in the literature originate from the crops or are a result of non-target wild plant contamination. Overall, I found that bees may be exposed to higher pesticide levels when foraging on crops close to the application timing and

may be exposed to more compounds when also foraging on wild plants, increasing the hazard from potential pesticide mixtures in their food.

Is there a relationship between the pesticide residues detected in nectar and pollen?

According to the systematic literature review of studies over the past 50 years (Chapter 2), pollen was the matrix most frequently evaluated and, of the compounds investigated, the majority were detected in pollen samples. The concentration of pesticides detected in pollen was higher than nectar and a strong positive correlation was observed between neonicotinoid residues in pollen and nectar of cultivated plant species; hence, risk assessments for neonicotinoids could be based on the concentrations found in pollen. The results from the 2019 study (Chapter 3) also showed a strong positive correlation between the overall azoxystrobin residues detected in pollen and those detected in nectar. Negative correlations were observed for the overall clothianidin and glyphosate residues, but these results would need further evaluation through a study that would warrant more detections.

Do the pesticide levels detected present a hazard for human health and terrestrial invertebrates?

Bee species can be exposed to high pesticide residues through plant pollen and nectar and honey bees can transfer these residues into their hives, contaminating honey and other bee products at concentrations potentially exceeding the established European maximum residue levels (MRLs) for honey and posing a hazard to human health. Moreover, my work has shown that bees can be exposed to residues (of all but one pesticide examined) that exceed the established European MRLs for Terrestrial invertebrate animals while foraging for pollen (Table 6.1).

Are there differences in the pesticide detections among the various pollen types evaluated?

According to 2020 results (Chapter 4) crop pollen was contaminated with fungicides but not insecticides, honey bees collected pollen mainly with fungicides, while bumble bee pollen had more insecticide detections. Most detections and highest number of compounds were observed in bumble bee collected pollen. The number of individual compound detections in bumble bee pollen increased when the number and percentage of wild plants in their pollen increased, a pattern that was not observed for honey bees. Given that no neonicotinoid residues were detected in crop pollen, these findings imply that bumble bee pollen contamination with neonicotinoids potentially originated from the wild plants growing near the crops, rather than the actual crops. These findings enhance the argument that different bee species may be

exposed to different compounds and raise concerns about whether honey bees are suitable surrogates for pesticide risk assessments for all bee species.

Table 6.1. Maximum pesticide residues detected in the present study, the respective matrix they were detected in, and maximum residue levels (MRLs) that are legally tolerated under the corresponding European Union regulation (EU). Detections in pollen and nectar that exceeded the MRLs are highlighted in bold.

Compound	Maximum residues detected (mg/kg)	Matrix detected	Maximum residue levels (mg/kg)		Regulation (EU)
			Honey and other apiculture products	Terrestrial invertebrate animals	
Acetamiprid	0.02	Bee pollen	0.05	0.02	2019/88
Azoxystrobin	0.03	Flower pollen	0.05	0.02	2022/476
Boscalid	10.4	Flower pollen	0.15	0.01	2022/1324
Clothianidin	0.01	Flower nectar	0.05	0.01	2017/671
Fluroxypyr	-	-	0.05	0.01	2021/1098
Glyphosate	0.21	Flower nectar	0.05	0.05	2013/293
Imidacloprid	0.02	Bee pollen	0.05	0.01	2021/1881
Prothioconazole	0.10	Flower pollen	0.05	0.01	2019/552
Thiacloprid	0.04	Bee pollen	0.20	0.01	2019/50
Thiamethoxam	0.02	Bee pollen	0.05	0.01	2017/671

6.3. Significance of findings

The EU has already one of the strictest regulatory systems in the world concerning the approval of pesticides (Handford et al., 2015). Through the Farm to Fork Strategy (European Commission, 2020c), the EU has set a very ambitious plan of a 50% pesticide reduction in overall use and risk of chemical pesticides, and a 50% reduction in the use of more hazardous pesticides to be achieved by 2030. My PhD thesis significantly contributes towards that cause by filling knowledge gaps, revealing the actual environmental contamination levels under real agricultural conditions, highlighting the potential hazard for bees and other pollinators, developing methods to facilitate future research on the exposure and exposure impact to pesticide residues via pollen and nectar, and ultimately by providing insights for improving the pesticide regulatory system for pollinator protection.

The EU biodiversity strategy for 2030 (European Commission, 2020d) through the Regulation on nature restoration (European Commission, 2022f), and the EU Pollinators Initiative (European Commission, 2023a) set the commitment to reverse the decline in wild pollinators by 2030. EU actions on pollinators aim to a) improve knowledge of pollinator decline, its causes and consequences, b) improve pollinator conservation and tackle the causes of pollinator decline, and c) mobilise society and promote strategic planning and cooperation at all levels. Since the excessive use of pesticides is a key driver of pollinator loss (IPBES, 2016), mitigating the impact of pesticide use on pollinators (i.e., through legal requirements to implement integrated pest management or through additional test methods for determining the toxicity of pesticides for pollinators, including sublethal and chronic effects), and reducing the risk and use of pesticides is a critical action towards that cause. Through my thesis, I provide a holistic approach to pesticide residue studies for the most exposure relevant matrices across all bee species, pollen and nectar. With the literature review I outlined the extent of pollen and nectar contamination levels and identified that while neonicotinoids are the most studied compounds for their residues in both crop and wild plants, the group of fungicides and herbicides, as well as product inert ingredients and metabolites of the active substances are severely understudied. Summarizing the minimum, maximum, and median residue concentrations retrieved for every compound detected to date in plant pollen and nectar across the globe, assists in the design of field-realistic studies on the impact of these pesticides, alone, as mixtures, or in combination with other stressors, on the various bee species. To simplify the residues studies since nectar is a matrix with many limitations in terms of its collection and chemical analysis, pollen was identified as a suitable surrogate for extrapolations of pesticide residue levels. Moreover, of all

the plant species evaluated model crops and wild plant species that could work as surrogates for identifying pesticide residues studies in pollen and nectar were determined.

The Member States are responsible for preparing national action plans on the correct use of pesticides, in line with the Sustainable Use of Pesticides Directive (European Commission, 2009a) and other relevant legislation, and for the verification of their enforcement. These should cover monitoring activities, training programs on the safe use of pesticides, licensing of pesticide handlers, and control measures. Specifically, through the Nature restoration law (European Commission, 2022f), Member States are obliged to reverse the decline of pollinators by 2030 by setting up robust monitoring schemes for pollinator conservation and by tackling the causes of their decline. The field samplings performed in this PhD research were my first attempt to apply the outcomes suggested by the preceding literature review. Ireland was used as an example of a European Member State, and I investigated the residues of the most relevant pesticide active substances in the most entomophilous crops and an abundant wild plant in Irish agricultural field margins. That was the first assessment of pollen and nectar contamination levels within Ireland, and the first evaluation of neonicotinoid residues under real agricultural conditions after their ban in Europe. I identified the most common compound mixtures that bees may encounter while foraging for pollen and nectar so that this information could be further evaluated in toxicity assays for lethal or sublethal effects on bees. Where harmful impacts on pollinators are found, legal measures will be taken to restrict or prohibit the use of the pesticides concerned (European Commission, 2023b). Glyphosate has been recognised as a very challenging pesticide compound in terms of chemical analysis, and for this reason, it was usually omitted from multiresidue analysis. With my thesis I managed to produce glyphosate and AMPA extraction protocols for both nectar and pollen and respective analytical methods, that could be adopted for future research. Moreover, the findings verified non-target plant pollen and nectar as a glyphosate source of contamination with uncertain direct repercussions to bees and consumption of honey and honey bee products by both bees and humans.

The presence of the neonicotinoid compounds several years after their last application and the persistence of some fungicides (e.g., boscalid), unveiled the long-term presence of some compounds that was found to be longer than initially expected from the literature, and pointed out that chronic exposure to pesticide mixtures is something that should be urgently incorporated in pesticide risk assessments for bees. The Commission aims to continue monitoring emergency authorisations granted by Member States for pesticides that are harmful to pollinators. Where emergency authorisations are not justified, the Commission will adopt decisions prohibiting Member States from repeating them (European Commission, 2023b). My

results raise serious concerns regarding the emergency authorisations of pesticide compounds such as the neonicotinoid insecticides, that should be taken into consideration during decision making.

With the revised EU Pollinators Initiative, the Commission aims to strengthen the current risk assessment of pesticides on bees and ensure that pesticides allowed in the EU do not pose a threat to various bee species (European Commission, 2023b). Through my PhD thesis I perform for the first time a comparison among the pollen from two crops and honey bee and bumble bee corbicular pollen, simultaneously collected from the same agricultural fields. The findings indicate that honey bees and bumble bees may be exposed to different pesticide categories, and my thesis highlights the need for more thorough pesticide risk assessments that also account for exposure of the various bee species to different pesticide categories.

6.4. Thesis limitations and sources of uncertainty

The main limitations of the work presented in this thesis relate to external parameters beyond my control (e.g., weather conditions), to the number of analytes evaluated, and the nature of the study itself, which was only focused on identifying general, yet representative, pollen and nectar contamination levels in field conditions, and did not include a complete assessment of the environmental fate of the pesticide compounds detected. More specifically:

1. During field work my sampling was opportunistic most of the time, and very much dependent on the weather conditions. This was mainly because the flowers had to be collected dry to avoid high humidity levels that could interfere with the final pesticide residues detected (especially in the case of nectar). Moreover, the pesticide applications were driven by weather forecasts that required dry weather for a certain amount of time prior and after the product applications and no windy conditions during the application - both rare climatic conditions for Ireland, even during the summer months.
2. There are more than 100 pesticide active substances in the Irish market and more than 1000 in the European Union. However, for my project I could only include 11 pesticides for residues in pollen and nectar analysis. Typically, broader analyses have higher limits of detection for individual compounds. In my work, it was critical to evaluate compounds at concentrations relevant to MRLs, so I chose to look at a subset of compounds with analysis methods capable of evaluating at lower concentrations. The evaluated compounds are indicative for each pesticide main category (fungicides, herbicides, and insecticides) and are the most relevant compounds in terms of their applied amount and frequency in arable

crops in Ireland. Yet, their selection was based on a report released in 2016, that has not been renewed since, so I could not have access to the most recent pesticide application data that would be even more realistic in time and space. Moreover, additional compounds were applied to broad bean and oilseed rape crops, most of which were not evaluated in the present study hence it is highly likely that we do not have a complete picture of the pesticide contamination. Ideally, each Member State should have an interactive digitised list of all the pesticide active and inert substances applied yearly with all the information related to their application and their main metabolites.

3. With my results I was able to make a preliminary first assessment of the pesticide contamination levels in pollen and nectar in Irish agricultural fields. However, to improve our understanding of the fate of sprayed pesticides in environmental matrices relevant to bees and be able to confidently generalise, model and make predictions, a ten-day sampling period with a daily interval, right after each pesticide application would be recommended.

6.5. Policy implications and recommendations for future work

Pollinator declines directly imperil the biodiversity equilibrium in our ecosystem, and indirectly jeopardise the future of sustainable food production. Current food production seems to be caught in a “vicious circle”: increasing pesticide use to increase yields, leading to worsening environmental degradation, and escalating health costs (Oliver et al., 2018). Agricultural pesticides, contamination of pollinator related matrices and food production form a complex network, linked to multiple and ambitious EU targets to be achieved over the next few decades (e.g., European Commission, 2020c, d). Overall, my research suggests that 1) long-term contamination of pollen and nectar with multiple pesticide compounds belonging to different pesticide categories is the rule rather than the exception under real agricultural conditions and 2) raises concerns on whether honey bees are suitable surrogates for pesticide risk assessments for all bee species. Since none of these are addressed in the current risk assessments for insect pollinators, my findings indicate the urgent need to re-assess and adapt the ecological risk assessment process for pesticide approval by making it more representative and more inclusive.

An integrated bee risk assessment, that evaluates the most common combinations of pesticide active or inert ingredients that bees may encounter on a field level (Gill et al., 2012; Sgolastra et al., 2020; Siviter et al., 2021), includes the mandatory initial evaluation of sublethal effects of all pesticide types (Sánchez-Bayo and Tennekes, 2017; Sgolastra et al., 2020), and that is specific to non-*Apis* bee species (i.e., bumble bees and solitary bees) (Franklin and Raine, 2019; Gradish

et al., 2019; Sgolastra et al., 2019; Willis Chan, 2020), would be highly recommended. We advise the toxicological evaluation of the several compound mixtures identified in the present study, to determine their impacts on foraging insects that may be exposed to them. Ecotoxicological tests on more than just one representative species should be preferred over speculations from predicted residue levels and standard ecotoxicological endpoints, since the latter do not account for bioavailability or indirect effects of pesticides (Hernández et al., 2020; FAO and ITPS, 2017; Silva, 2022; Topping et al., 2020). Knowledge of toxicity should be expanded to more wild bee species and respective LD₅₀ and LDD₅₀ values should be established for fungicide and herbicide compounds. Assessments should also cover simultaneous and repeated exposure patterns of pesticides in their test designs (Sybertz et al., 2020; Weisner et al., 2021).

Approved substances are re-evaluated by the European Commission after a maximum of 10 or 15 years (for new or already existing active substances, respectively), using the pre-approval procedure. Restrictions to substances may occur before that if enough evidence justifies it (European Commission, 2013a). Most post-approval surveillance data is however linked to residues testing in food (Zeitlin et al., 2021). An initial risk assessment is hardly ever adequate, since the same pesticide products will be released in different parts of the world, under different climatic conditions and their environmental fate will likely differ from the one preliminary predicted (Humann Guilleminot et al., 2019). Requirements for pesticide approval must be re-evaluated, especially in relation to longer pesticide persistence, hazard gaps and the lack of data on environmental mixtures (Silva, 2022). Despite the precautionary principal foundation of the EU regulation, there are high-risk substances in the EU market (e.g., glyphosate, which is candidate for substitution), as well as substances with known data gaps (e.g., boscalid). In advance of, and to inform the upcoming renewal of market authorization for glyphosate in the European Union, our results highlight the need for immediate investigation of glyphosate as desiccant before harvesting crops, to elucidate the fate of glyphosate residues in soil and non-target flowering plants growing near crop fields, over a period of several days after the desiccant spraying.

In addition, the number of special use or emergency authorizations of banned or not yet approved pesticides is increasing over the years (PAN, 2011), suggesting misuse of the derogation clause. Most pollen and nectar samples analysed in this thesis contained mixtures of pesticide residues, and some compounds were found to be more persistent than assumed earlier in the dossier's legislation studies and literature (e.g., neonicotinoids and boscalid). These findings raise concerns on the current process of making assumptions of pesticide use and half-life times. There is a need for constant post-registration monitoring to determine

whether risk assessment assumptions are met under real agricultural conditions and identify any unforeseen adverse effects (Sgolastra et al., 2020; Siviter and Muth, 2020). In the present study, the detections of neonicotinoid pesticides in bee related matrices several years after their outdoor ban in Europe, and after their actual application on the fields, are of concern, and suggest that continuous monitoring of neonicotinoid presence and fate in the field environment is warranted. The role of pesticide production companies in the risk assessment process, and the feasibility to create an organisation that will assess pesticide compounds independently and impartially of economic interests, should also be considered.

Apart from the risk assessments that are specifically designed for insect pollinators, there are several other indirect steps to be made to ensure a holistic approach towards safeguarding pollinators from pesticide use in agriculture. Agricultural area represents 42% of the EU total land area (European Commission, 2018b), thus pesticide monitoring is a key piece in environmental management strategies. Exposure through contaminated pollen and nectar is considered pivotal across all bee species and these matrices are preferred for testing in bee toxicological assays for pesticide risk assessment. Thus, for an accurate representation of the actual risk, it is necessary to use concentrations that a bee would encounter in a real agricultural setting while foraging. Ideally, pollen and nectar from crops and wild plants growing in agricultural areas should be assessed for pesticide residue levels, by evaluating samples collected on the day and for day days after the initial pesticide application on the crops (Kyriakopoulou et al., 2017). Soil sampling should be performed simultaneously with the respective pollen and nectar sampling. This would enable accurate assumptions about the environmental fate of pesticide compounds that could be incorporated in modelling predictions. This could be incorporated in monitoring at a smaller scale (e.g., few representative fields every four years), however, it is something very time consuming and would increase the monitoring costs. Since many of the pesticide detections originated from fields where the compounds were not recently applied, and due to their systemic nature, it could be assumed that contaminations, likely occurred through soil translocation in most cases. Soil is also a very relevant matrix considering pesticide exposure for several non-*Apis* bee species (Boyle et al., 2019; Gradish et al., 2019; Rondeau et al., 2022; Willis Chan et al., 2019; Willis Chan and Raine, 2021). As most of the pesticide-soil data originate from individual scientific studies (Sabzevari and Hofman, 2022), it is necessary to adopt a better and continuous soil monitoring program for agricultural pesticide residues are urgently needed (Silva, 2022). Such information would contribute to establishing a pollution baseline and evaluating the efficiency of pesticide reduction schemes.

Despite the limitations due to the relatively high costs of pesticide analyses, for a high quality and realistic risk assessment, monitoring programs should assess all past and currently used pesticide active substances, but also metabolites and inert ingredients, and not restricted to certain matrices and substances (ECA, 2020). Despite the analytical challenges associated with implementing methods with very specific and expensive associated costs, it is important that this process is continuous and harmonised across all EU Member States, while adhering to the EU regulatory targets, which should be constantly updated according to new scientific findings. Sampling and analytical methods should be harmonised, and all monitoring data should be centralized and available in IPCHEM portal (Information Platform for Chemical Monitoring, <https://ipchem.jrc.ec.europa.eu/>). It is important though to ensure that the analytical methods deployed have sensitive enough LODs, and LOQs below the relevant MRLs to enable accurate and realistic reporting for subsequent decision making. Detailed and up to date pesticide use data are needed for the interpretation of the residue results, the risk prediction, and measure suggestions. Limited access to detailed and homogenous pesticide use data has been an issue reported before (López-Ballesteros et al., 2022; Mesnage et al., 2021; Silva, 2022). Transparency and easy access to pesticide data from industry and regulators must be strongly encouraged and facilitated. Farmers should be encouraged to be more actively involved in the monitoring process, rather than doubting and feeling insecure about sharing their data. Consumers and producers must change their perspectives on what constitutes a pleasing and safe for consumption food product, so that pesticide applications are strictly limited to those needed for enhancing crop yields and are not driven by aesthetics (Bloom et al., 2021).

There is clear evidence that pesticides, especially insecticides, are a major pressure on wild pollinators, as well as the lack of wildflowers linked to herbicide use. Therefore, implementing Integrated pest management (IPM) and organic farming (if done on a large scale), with respectively reduced and no synthetic pesticides input on farms are likely to benefit wild pollinator populations (Andersson et al., 2013, 2014; Austin et al., 2019; Catarino et al., 2019; Garratt et al., 2018). The Commission will also help ensure the implementation of IPM and improve the harmonised risk indicators to reduce potential impacts of approved pesticides on pollinators (European Commission, 2023b). IPM systems and organic farming systems include several farming practices that are likely to increase wild pollinator abundance, including: reduced or zero pesticide use, cover crops and green manure crops, tolerance of weeds, high densities of field margins and landscape features, diverse crop rotations, and a diverse mix of crops, grassland, and woody vegetation (Mottershead and Underwood, 2020). IPM has been compulsory in the EU since 2014 (European Commission, 2009a), and organic production is

highly encouraged, and expected to represent at least 25% of the EU's agricultural land by 2030 (European Commission, 2020c). Nevertheless, even IPM and organically managed areas can be affected by pesticides, due to current and/or past use of pesticides, and off-site contamination (Fagan et al., 2020; Geissen et al., 2021; Riedo et al., 2021). More discussion and insights on the benefits versus the adverse effects of pesticide use are therefore urgently needed to transition to a safer, more sustainable, and resilient pesticide use (Gladek et al., 2017).

Reducing the use of pesticides is needed to promote and safeguard the delivery of pollination services. In tandem with the constantly increasing and rapidly changing agricultural demands, it appears necessary to obtain adaptable, efficient, and reliable ecological risk assessments for pollinator protection from pesticides. A refined, universal, and multifaceted approach including scientists, researchers, farmers, consumers, pesticide manufacturers, and policymakers working altogether is needed to promote a future with sustainable pesticide use that does not imperil pollinator health and the delivery of pollination services.

7. References

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8. Appendices

Appendix A - Supplementary data for Chapter 2

Table A1. The plant taxa evaluated for PPP residues in pollen and nectar, their synonyms, varieties, and common names.

Plant family	Plant species	Synonyms	Variety	Common name
Amaryllidaceae	<i>Allium cepa</i> L.			Onion
Apiaceae	<i>Aethusa cynapium</i> L.			Fool's parsley
	<i>Anthriscus sylvestris</i> (L.) Hoffm.			Cow Parsley
	<i>Heracleum sphondylium</i> L.			Hogweed
	<i>Pimpinella saxifraga</i> L.			Burnet-saxifrage
Aquifoliaceae	<i>Ilex x attenuata</i>			Foster holly
Asparagaceae	<i>Muscari armeniacum</i> Leichtlin ex Baker			Grape hyacinth
Asteraceae	<i>Ageratum houstonianum</i> Mill.			Floss flower
	<i>Arctium</i> sp.			Burdock
	<i>Carduus</i> sp.			Thistle
	<i>Centaurea nigra</i> L.			Common knapweed
	<i>Centaurea scabiosa</i> L.			Greater knapweed
	<i>Cirsium arvense</i> (L.) Scop.			Thistle
	<i>Cirsium vulgare</i> (Savi) Ten.			Thistle

<i>Cosmos bipinnatus</i> Cav.		'Casanova Violet'	Mexican aster
<i>Coreopsis grandiflora</i> Sweet		'Early Sunrise'	Large-flowered tickseed
<i>Dahlia x hortensis</i> Guillaumin		'Bishop of Llandaff'	Dahlia
<i>Dahlia x hybrida</i> (1)		'Gallery Art Fair'	Dahlia
<i>Dahlia x hybrida</i> (2)		'Mystic Dreamer'	Dahlia
<i>Helianthus annuus</i> L.			Sunflower
<i>Hieracium</i> sp.			Hawkweed
<i>Jacobaea vulgaris</i> Gaertn.	<i>Senecio jacobaea</i> L.		Ragwort
<i>Matricaria discoidea</i> DC.			Wild chamomile
<i>Matricaria chamomilla</i> L.	<i>Matricaria recutita</i> L.		Chamomile
<i>Sonchus arvensis</i> L.			Sow thistle
<i>Sonchus oleraceus</i> L.			Sow thistle
Unidentified Asteraceae			

Boraginaceae	<i>Myosotis arvensis</i> (L.) Hill		Field forget-me-not
	<i>Brassica napus</i> L. (1)	Spring	Oilseed rape
	<i>Brassica napus</i> L. (2)	Winter	Oilseed rape
Brassicaceae	<i>Brassica rapa</i> (L.) L.		Field mustard
	<i>Cardamine pratensis</i> L.		Cuckooflower
	<i>Erysimum linifolium</i> (Pers.) J. Gay	'Bowles's Manve'	Wallflower
Campanulaceae	<i>Campanula portenschlagiana</i> Schult.		Dalmatian bellflower
Caprifoliaceae	<i>Scabiosa columbaria</i> L.	'Pink Mist'	Scabious 'Pink Mist'

	<i>Silene dioica</i> (L.) Clairv.		Red campion
Caryophyllaceae	<i>Silene latifolia</i> Poir.		White campion
	<i>Silene vulgaris</i> (Moench) Garcke		Bladder campion
Clethraceae	<i>Clethra alnifolia</i> L.	Sixteen candles	Summersweet
	<i>Calystegia sepium</i> (L.) R. Br.		Heavenly trumpets
Convolvulaceae	<i>Calystegia silvatica</i> (Kit.) Griseb.		Large bindweed
	<i>Convolvulus arvensis</i> L.		Field bindweed
	<i>Bryonia dioica</i> Jacq.		English mandrake
Cucurbitaceae	<i>Cucurbita pepo</i> L. (1)	Howden'	Squash
	<i>Cucurbita pepo</i> L. (2)	Multipik'	Squash
	<i>Cucurbita pepo</i> L. (3)	Sunray'	Squash
	<i>Cucurbita pepo</i> L. (4)	Bush Delicata'	Squash
Dipsacaceae	<i>Knautia arvensis</i> (L.) DC.		Field scabious
Ericaceae	<i>Erica carnea</i> L.		Winter heath
	<i>Medicago sativa</i> L.		Alfalfa
Fabaceae	<i>Trifolium pratense</i> L.		Red clover
	<i>Trifolium repens</i> L.		White clover
	<i>Vicia faba</i> L.		Field bean
	<i>Vicia sativa</i> L.		Common vetch
Hypericaceae	<i>Hypericum hirsutum</i> L.		Square-stalked St John's-wort
Iridaceae	<i>Crocus vernus</i> (L.) Hill	'Golden Yellow'	Crocus

	<i>Iris tingitana</i> Boiss. & Reut. × <i>Iris xiphium</i> L.		Morocco iris
	<i>Ballota nigra</i> L.		Black horehound
	<i>Glechoma hederacea</i> L.		Ground-ivy
	<i>Lamium album</i> L.		White dead-nettle
	<i>Lamium purpureum</i> L.		Red dead-nettle
Lamiaceae	<i>Lavandula stoechas</i> L.	'Victory'	French lavender
	<i>Nepeta cataria</i> L. (1)	'Six Hill Giant'	Catnip
	<i>Nepeta cataria</i> L. (2)	'Walkers Low'	Catnip
	<i>Salvia longispicata</i> M. Martens & Galeotti x <i>Salvia farinacea</i> Benth.	'Mystic Spires'	Sage
	<i>Stachys sylvatica</i> L.		Hedge woundwort
	<i>Gossypium</i> sp.		Upland cotton
Malvaceae	<i>Malva moschata</i> L.		Musk mallow
	<i>Malva sylvestris</i> L.		Common mallow
Oleaceae	<i>Ligustrum vulgare</i> L.		European privet
	<i>Epilobium hirsutum</i> L.		Hairy willowherb
Onagraceae	<i>Fuchsia</i> sp.		Fuchsia
	<i>Fumaria officinalis</i> L.		Drug fumitory
Papaveraceae	<i>Papaver rhoeas</i> L.		Common poppy
	<i>Digitalis purpurea</i> L.	'Dalmatian White'	Foxglove
Plantaginaceae	<i>Microrrhinum minus</i> (L.) Fourr.	<i>Chaenorhinum minus</i> (L.) Lange	Dwarf snapdragon
	<i>Veronica persica</i> Poir.		Common field-speedwell

	<i>Veronica spicata</i> L.		Spiked speedwell
Poaceae	<i>Zea mays</i> L.		Maize
Ranunculaceae	<i>Clematis vitalba</i> L.		Old man's beard
	<i>Ranunculus repens</i> L.		Creeping buttercup
Rosaceae	<i>Agrimonia eupatoria</i> L.		Stickwort
	<i>Fragaria x ananassa</i> (Duchesne ex Weston) Duchesne ex Rozier	'Toscana F1'	Strawberry
	<i>Rosa arvensis</i> Huds.		Wild rose
	<i>Rosa canina</i> L.		Wild rose
	<i>Rubus fruticosus</i> agg.		Bramble
	<i>Rubus</i> sp.		Raspberry
Rutaceae	<i>Citrus x sinensis</i> (1)	Valencia	Orange
	<i>Citrus x sinensis</i> (2)	Navel	Orange
	<i>Citrus reticulata</i> Blanco		Tangerine
Solanaceae	<i>Datura stramonium</i> L.		Devil's snare
	<i>Solanum dulcamara</i> L.		Bittersweet
Tropaeolaceae	<i>Tropaeolum</i> sp.		Nasturtium
Violaceae	<i>Viola arvensis</i> Murray		Field pansy

Table A2.1. The daily Maximum Residue Uptake (MRU) for honey bees and bumble bees.

Compound	Maximum residues (ng/g)		Honey bees				Bumble bees			
			<i>Apis</i> spp.				<i>Bombus</i> spp.			
			MRU/day (µg ai)		LD ₅₀		MRU/day (µg ai)		LD ₅₀	
	Pollen	Nectar	Pollen (nurse)	Nectar (forager)	C ¹	O ²	Pollen	Nectar	C	O
5-Hydroxy-imidacloprid	-	69								
Acetamiprid	0.82	7.6	0.00001	0.002	8.09	14.53	0.00002	0.002	>100	22.2
Boscalid	38	2.05	0.0004	0.0005	>200	>166				
Carbendazim	204	1.25	0.002	0.0003	>50	>756				
Carbofuran	1400	-	0.01	-	0.036	0.05				
Carboxin	0.06	-	0.000001	-	>100	>100				
Chlorothalonil	422	76	0.04	0.02	>40	>40	0.01	0.02	>100	>94
Chlorpyrifos	163	-	0.002	-	0.059	0.25	0.005	-	>1.58	0.23
Clothianidin	11	2992	0.0001	0.7	0.044	0.004	0.0003	0.95	0.02	
Cypermethrin	58.5	-	0.001	-	0.023	0.172	0.002	-	0.119	0.263
Dimethoate	500	22940	0.005	5.3	0.10	0.10	0.01	7.27	>0.94	>0.33
Dinotefuran	88.3	9.2	0.001	0.002	>0.023					
Epoxiconazole	31	-	0.0003	-	>100	>83	0.001		>200	>165.5
Fluoxastrobin	0.15	0.01	0.000001	0.000002	>200	>843	0.000004	0.000003	>100	>209
Flusilazole	16	-	0.0002	-	165.0	33.8				

Imidacloprid	150	6588	0.001	1.51	0.081	0.0037	0.004	2.09	0.218	0.038
Imidacloprid olefin	-	55	-	-						
Iprodione	995	18.5	0.01	0.004	>100	>100				
Lambda-cyhalothrin	57.51	-	0.0005	-	0.038	0.91	0.002		0.11	0.16
Metalaxyl	3.1	-	0.00003	-	200	269				
Metconazole	19	-	0.0002	-	>100	85	0.001		>100	111.10
Oxamyl	35	35	0.0003	0.01	0.47	0.38	0.001	0.01	39.30	0.36
Phorate	-	100	-	0.02	0.32	>0.43		0.03	>1	
Piperonyl butoxide	0.36	-	0.000003	-	294.0					
Prochloraz	46	0.3	0.0004	0.0001	141.3	>101				
Pyraclostrobin	19.6	0.6	0.0002	0.0001	>100	>110	0.001	0.0002	>100	>97.2
Silthiofam	0.12	-	0.000001	-	>100	>105.7				
Spinosad	320	-	0.003	-	0.0036	0.0057				
Spiroxamine	328	0.15	0.003	0.00003	4.20	>100				
Tebuconazole	34	0.18	0.0003	0.00004	>200	>83.05				
Thiacloprid	78	65.6	0.001	0.02	38.82	17.32	0.002	0.02	>100	
Thiamethoxam	95.2	11	0.001	0.003	0.024	0.005	0.003	0.003	0.028	0.005
Trifloxystrobin	104	1.2	0.001	0.0003	>100	>110				
Triticonazole	0.12	-	0.000001	-	>100	>92.26				

^{1,2} Contact and Oral LD₅₀S.

Table A2.2. The daily Maximum Residue Uptake (MRU) for solitary bees.

			Solitary bees									
			<i>Osmia</i> sp.		<i>Megachile</i> rotundata		<i>Scaptotrigona postica</i>	<i>Nannotrigona perilampoides</i>	<i>Trigona</i> sp.	<i>Nomia melanderi</i>		
Compound	Maximum residues (ng/g)		MRU/day (µg ai)		LD ₅₀							
	Pollen	Nectar	Pollen	Nectar	C ¹	O ²	C	O	O	C	C	C
5-Hydroxy-imidacloprid	-	69										
Acetamiprid	0.82	7.6	0.00001	0.001	1.72							
Boscalid	38	2.05										
Carbendazim	204	1.25										
Carbofuran	1400	-										
Carboxin	0.06	-										
Chlorothalonil	422	76										
Chlorpyrifos	163	-	0.002		4.19							
Clothianidin	11	2992	0.0001	0.40	0.10	>8.4		0.08				
Cypermethrin	58.5	-	0.001								0.07	
Dimethoate	500	22940	0.01	3.07	1.02	0.25	0.0009					0.0021
Dinotefuran	88.3	9.2										
Epoxiconazole	31	-										
Fluoxastrobin	0.15	0.01										
Flusilazole	16	-										
Imidacloprid	150	6588	0.002	0.88	0.031		0.17		24.50			
Imidacloprid olefin	-	55										
Iprodione	995	18.5	0.01	0.002	>125	>125						
Lambda-cyhalothrin	57.51	-	0.001	-	0.136							

Metalaxyl	3.1	-						
Metconazole	19	-						
Oxamyl	35	35						
Phorate	-	100						
Piperonyl butoxide	0.36	-						
Prochloraz	46	0.3						
Pyraclostrobin	19.6	0.6						
Silthiofam	0.12	-						
Spinosad	320	-						
Spiroxamine	328	0.15						
Tebuconazole	34	0.18						
Thiacloprid	78	65.6	0.001	0.009	1.16		0.007	
Thiamethoxam	95.2	11	0.001	0.001			0.004	0.0051
Trifloxystrobin	104	1.2						
Triticonazole	0.12	-						

^{1,2} Contact and Oral LD₅₀s.

Table A3. The Maximum Residue Uptake (MRU) per one foraging bout of a nectar foraging honey bee and the respective values of Hazard Quotient (HQ) and Estimated % LD₅₀.

Nectar foraging honey bees					
Compound	MRU/foraging bout (µg ai)	Contact		Oral	
		HQ	% LD ₅₀	HQ	% LD ₅₀
Acetamiprid	0.41	0.94	5.01	0.52	2.79
Boscalid	0.11	0.01	0.05	0.01	0.07
Carbendazim	0.07	0.03	0.13	0.00	0.01
Chlorothalonil	4.05	1.90	10.13	1.90	10.13
Clothianidin	159	68000	362440	748000	3986840
Dimethoate	1223	229400	1222702	229400	1222702
Dinotefuran	0.49	400	2132		
Fluoxastrobin	0.001	0.0001	0.0003	0.00001	0.0001
Imidacloprid	351	81333	433507	1780541	9490281
Imidacloprid olefin	2.93	-	-		
Iprodione	0.99	0.19	0.99	0.19	0.99
Oxamyl	1.87	74.47	397	92.11	491
Phorate	5.33	312.50	1666	232.56	1240
Prochloraz	0.02	0.002	0.01	0.003	0.02
Pyraclostrobin	0.03	0.01	0.03	0.01	0.03
Spiroxamine	0.01	0.04	0.2	0.002	0.01
Tebuconazole	0.01	0.001	0.005	0.002	0.01
Thiacloprid	3.50	1.69	9.01	3.79	20.19
Thiamethoxam	0.59	458.33	2443	2200	11726
Trifloxystrobin	0.06	0.01	0.06	0.01	0.06

Table A4. The citations of the 25 papers included in the present review.

Number	Citation
1	Arceo-Gómez et al. 2018
2	Bailey et al. 2005
3	Barker et al. 1980
4	Bonmatin et al. 2003
5	Bonmatin et al. 2005
6	Botías et al. 2015
7	Byrne et al. 2014
8	David et al. 2015
9	David et al. 2016
10	Dively and Kamel 2012
11	Henry et al. 2015
12	Jiang et al. 2018
13	Krupke et al. 2012
14	Larson et al. 2013
15	Larson et al. 2015
16	Laurent et al. 2013
17	Lentola et al. 2017
18	Lord et al. 1968
19	Mach et al. 2018
20	Pohorecka et al. 2012
21	Schmuck et al. 2001
22	Stoner and Eitzer 2012
23	Thompson et al. 2015
24	Waller and Barker 1979
25	Xu et al. 2016

Table A5. The results of the Generalised Linear Mixed Model analysis.

	Estimate	Standard Error	z value	Pr (> z)
Intercept (Clothianidin-Cultivated)	-0.07	0.367	-0.191	0.849
Ln (Pollen) (Cultivated)	0.593	0.084	7.063	<0.001
Status - Wild	-2.751	0.397	-6.929	<0.001
Dinotefuran	-0.217	0.423	-0.513	0.608
Imidacloprid	-0.291	0.167	-1.745	0.081
Thiacloprid	-1.498	0.168	-8.945	<0.001
Thiamethoxam	-0.097	0.196	-0.496	0.62
Ln (Pollen) (Wild)	-0.469	0.092	-5.071	<0.001

Appendix B - Supplementary data for Chapter 3

Table B1. List with all the chemical interventions in the ten *Brassica napus* (oilseed rape or canola) crop fields during the cropping period (2018-2019). The pesticide applications of the compounds evaluated in the present study are highlighted in bold.

Field	Date	Method	BBCH scale ¹	Type	Product	Systemic	Active ingredient	Concentration	Application rate	Formulation type
1	11/Jul/19	Spray	GS86	Herbicide	Roundup Gold 450®	Yes	Glyphosate	450 g/L	1.5 L/ha	Soluble liquid
				Herbicide	Cleranda®	Yes	Imazamox Metazachlor	17.5 g/L 375 g/L	1.5 L/ha	Suspension concentrate
	15/Sep/18	Spray	GS10-18				Fatty acid esters	406 g /L	0.5 L/ha	Emulsifiable concentrate
				Adjuvant	Dash HC®	Yes	Alkoxyated alcohols- phosphate esters	244 g /L	0.5 L/ha	Emulsifiable concentrate
2	25/Feb/19	Spreader	GS50	Fertilizer	Nitrogen Phosphorus Potassium		N-P-K	10, 10, 20	3 bags/acre	
	1/Apr/19		GS63	Fertilizer	Sulphur		Urea	7% urea	2.5 bags/acre	
	10/Apr/19	Spray	GS65	Fungicide	Proline®	Yes	Prothioconazole	250 g/L	3 L/ha	Emulsifiable concentrate
	10/Jul/19	Spray	GS86	Herbicide	Roundup PowerMax®	Yes	Glyphosate	720 g/L	20 kg/ha	Soluble liquid
	2/Sep/18	Spray	GS00	Herbicide	Butisan S®	Yes	Metazachlor	500 g/L	1.5 L/ha	Suspension concentrate
3	5/Sep/18		GS09	Molluscicide	Adama Gusto®	Yes	Metaldehyde	3% w/w		Peletted bait
	20/Sep/18	Spray	GS12	Fungicide	Proline®	Yes	Prothioconazole	250 g/L	0.5 L/ha	Emulsifiable concentrate

	10/Jan/19	Spray	GS39	Herbicide	Proper Flo®	Yes	Propyzamide	400 g/L	1.5 L/ha	Suspension concentrate
	20/Feb/19	Spray	GS50	Fungicide	Proline®	Yes	Prothioconazole	250 g/L	0.5 L/ha	Emulsifiable concentrate
				Trace element	Bortrac		Boron	150 g/L	2 L/ha	True liquid
	23/Feb/19	Spreader	GS50	Fertilizer	Nitrogen Phosphorus Potassium		N-P-K	5,6,35	245 kg/L	
	9/Mar/19	Spreader	GS59	Fertilizer	Ammonium Sulphate Nitrate		A-S-N	14,26	255 kg/L	
	21/Mar/19	Spray	GS60	Fungicide	Proline®	Yes	Prothioconazole	250 g/L	0.35 L/ha	Emulsifiable concentrate
	21/Mar/19	Spray		Fungicide	Caramba®	Yes	Metconazole	60 g/L	0.4 L/ha	Emulsifiable concentrate
	17/Aug/18	Spray	-	Herbicide	Roundup Gold 450®	Yes	Glyphosate	450 g/L	1.5 L/ha	Soluble liquid
4	20/Nov/18	Spray	GS19-23	Fungicide	Proline®	Yes	Prothioconazole	250 g/L	0.4 L/ha	Emulsifiable concentrate
	1/Dec/18	Spray	GS19-23	Fungicide	Riza®	Yes	Tebuconazole	250 g/L	0.7 L/ha	Emulsifiable concentrate
	30/Aug/18	Seed treatment	GS00	Fungicide	TMTD 98% SATEC®	No	Thiram	980 g/kg	4 g/seed	
	15/Aug/18	Spreader	-	Fertilizer	Chicken manure					
5				Herbicide	Falcon®	Yes	Propaquizafop	100 g/L	0.5 L/ha	Emulsifiable concentrate
	17/Sep/18	Spray	GS19	Herbicide	Katamaran Turbo®	Yes	Metazachlor Quinmerac	375 g/L 125 g/L	2.5 L/ha	Suspension concentrate

6	10/Nov/18	Spray	GS19-23	Fungicide	Proline®	Yes	Prothioconazole	250 g/L	0.4 L/ha	Emulsifiable concentrate
				Trace element	Bortrac		Boron	150 g/L	2.5 L/ha	True liquid
	3/Mar/19	Spreader	GS59	Fertiliser	Nitrogen Phosphorus Potassium and Sulphate		N-P-K-S	9, 7.4, 25, 2	495 kg/ha	
	12/Mar/19	Spray	GS61	Fungicide	Folicur (Generic riza)®	Yes	Tebuconazole	250 g/L	0.66 L/ha	Emulsifiable concentrate
				Trace element	Bortrac		Boron	150 g/L	1 L/ha	True liquid
	21/Apr/19	Spray	GS63	Fungicide	Amistar (Zoxis)®	Yes	Azoxystrobin	251 g/L	0.6 L/ha	Suspension concentrate
	4/May/19	Spray	GS65	Fungicide	Filan®	Yes	Boscalid	500 g/L	0.5 L/ha	Water dispersible granules
	4/May/19	Spray	GS67	Fertiliser	Magnitech		Zinc, Nitrogen, Manganese, Magnesium, Iron		1 L/ha	
	16/Jul/19	Spray	GS86	Herbicide	RoundUp Powermax®	Yes	Glyphosate	720 g/kg	1.67 kg/ha	Soluble liquid
				Adjuvant	Iskay®		Styrene-butadiene copolymer blend		8 L/ha	
6	27/Mar/19	Seed treatment	GS00	Fungicide	TMTD 98% SATEC®	No	Thiram	980 g/kg	4 g/seed	
	10/May/19	Spray	GS10-18	Herbicide	Cleranda®	Yes	Imazamox Metazachlor	17.5 g/L 375 g/L	2 L/ha	Suspension concentrate
				Adjuvant	Dash®	Yes	Fatty acid esters	348.75 g/L	1 L/ha	Emulsifiable concentrate

							Alkoxylated alcohols- phosphate esters	209.25 g/L	1 L/ha	Emulsifiable concentrate			
							Fertilizer	Bortrac	Boron	150 g/L	2 L/ha	True liquid	
							Fungicide	Proline®	Yes	Prothioconazole	250 g/L	0.4 L/ha	Emulsifiable concentrate
							Trace element	Mantrac	Magnese		0.4 L/ha	Flowable liquid	
7	20/May/19	Spray	GS50	Trace element	Bortrac		Boron	150 g/L	3 L/ha	True liquid			
	10/Apr/19	Spray		GS00	Herbicide	Crusader®	Yes	Pyroxsulam	30 g/L	4 kg/ha	Oil dispersion		
	15/Apr/19	Spray		GS10	Herbicide	Stratos®	Yes	Cycloxydim	100 g/L	1.5 L/ha	Emulsifiable concentrate		
8	23/May/19	Spray	GS10-18	Herbicide	Fusilade Max®	Yes	Fluazifop-P-butyl	125 g/L	1.5 L/ha	Emulsifiable concentrate			
	23/Jun/19	Spray	GS67	Fungicide	Prosaro®	Yes	Prothioconazole Tebuconazole	125 g/L	1 L/ha	Emulsifiable concentrate			
	10/Apr/19	Seed treatment	GS00	Fungicide	TMTD 98% SATEC®	No	Thiram	980 g/kg	4 g/seed				
9	10/May/19	Spreader	GS15	Fertilizer	YaraBela SULPHAN®		Nitrogen Sulphur	24% N 15% SO3					
	17/May/19	Spray	GS20	Herbicide	Falcon®	Yes	Propaquizafop	100 g/L	0.8 L/ha	Emulsifiable concentrate			
	13/Apr/19	Seed treatment	GS00	Fungicide	TMTD 98% SATEC®	No	Thiram	980 g/kg	4 g/seed				
10	9/Mar/19	Spray	-	Herbicide	Mizr XL®	Yes	Glyphosate	360 g/L	2.5 L/ha	Soluble liquid			
	6/Apr/19	Seed treatment	GS00	Fungicide	TMTD 98% SATEC®	No	Thiram	980 g/kg	4 g/seed				
	20/May/19	Spray	GS10-18	Herbicide	Cleranda®	Yes	Imazamox Metazachlor	17.5 g/L 375 g/L	1.8 L/ha	Suspension concentrate			

			Adjuvant	Dash®	Yes	Fatty acid esters Alkoxylated alcohols- phosphate esters	348.75 g/L 209.25 g/L	1.3 L/ha	Emulsifiable concentrate
27/May/19	Spray	GS50	Fungicide	Proline®	Yes	Prothioconazole	250 g/L	0.4 L/ha	Emulsifiable concentrate
			Trace element	Bortrac		Boron	150 g/L	3 L/ha	True liquid
			Trace element	Mantrac		Magnese		0.4 L/ha	Flowable liquid
			Magnesium	Epsom salts		Magnesium sulfate		2.5 L/ha	
3/Jul/19	Spray	GS65	Fungicide	Proline®	Yes	Prothioconazole	250 g/L	0.4 L/ha	Emulsifiable concentrate
			Fertilizer	Magnus		Magnesium Sulphur	480 g/L 360 g/L	0.9 L/ha	

¹ The BBCH scale is used to identify the phenological development stages of the crops according to BBCH Monograph (see references Meier, 2001).

Table B2. Information related to the ten *Brassica napus* fields (location, size, crop variety, sowing date, adjacent fields) and information regarding the sampling (date of sampling, mean temperature recorded, plant species sampled).

Field	County	Variety	Sowing	Adjacent fields	Temperature (°C)	Sampling date	Plant species
1	Wicklow	DK ¹ Exception	27/Aug/18	Grass	4	31/Mar/19	<i>B. napus</i>
					17	13/Jul/19	<i>R. fruticosus</i>
2	Wicklow	Phoenix CL ²	24/Aug/18	Crop	5	2/Apr/19	<i>B. napus</i>
					17	13/Jul/19	<i>R. fruticosus</i>
					19	15/Jul/19	<i>R. fruticosus</i>
3	Wexford	Elgar	01/Sep/18	Crop	9	28/Mar/19	<i>B. napus</i>
					6	13/Apr/19	<i>B. napus</i>
4	Wicklow	DK ¹ Exception	21/Aug/18	Crop	8	06/Apr/19	<i>B. napus</i>
					15	06/Jul/19	<i>R. fruticosus</i>
5	Kildare	DK ¹ Exception	30/Aug/18	Grass	7	11/Apr/19	<i>B. napus</i>
					12	11/May/19	<i>B. napus</i>
					18	18/Jul/22	<i>R. fruticosus</i>
					20	23/Jul/22	<i>R. fruticosus</i>
6	Kildare	Hybrid	27/Mar/19	Grass	16	17/Jun/19	<i>B. napus</i>
					23	09/Jul/19	<i>R. fruticosus</i>
7	Kildare	Lumen	10/Apr/19	Grass	17	26/Jun/19	<i>B. napus</i>
					23	09/Jul/19	<i>R. fruticosus</i>
8	Carlow	Lumen	10/Apr/19	Grass	19	26/Jun/19	<i>B. napus</i>
9	Wicklow	Lumen	13/Apr/19	Crop	14	27/Jun/19	<i>B. napus</i>
					15	08/Jul/19	<i>R. fruticosus</i>
10	Kildare	Hybrid	06/Apr/19	Crop	16	04/Jul/19	<i>B. napus</i>
					20	09/Jul/19	<i>R. fruticosus</i>

¹ DK refers to the hallmark of DeKalbs®

² CL refers to the hallmark of Clearfield®

Table B3. Retention times and optimised LC-MS/MS acquisition parameters for the pesticide compounds and their internal standards.

Compound	Formula	ESI mode	Q (m/z)	CE	q (m/z)	CE	Rt (min)
Neonicotinoids							
Thiamethoxam	C ₁₀ H ₁₁ ClN ₄	+	292>211.1	15	292>181	24	3.442
Clothianidin	C ₆ N ₅ H ₈ SO ₂ Cl	+	250>169	12	250>132	12	4.027
Imidacloprid	C ₉ H ₁₀ ClN ₅ O ₂	+	256.2>175.2	25	256.2>209	20	4.342
Acetamiprid	C ₁₀ H ₉ ClN ₄ S	+	223.2>126.1	20	223.2>56.1	20	4.609
Thiacloprid	C ₈ H ₁₀ ClN ₅ O ₃ S	+	253>126	36	253>90	50	5.573
Herbicide							
Fluroxypyr	C ₇ H ₅ Cl ₂ FN ₂ O ₃	+	255>209	12	255>101	24	6.054
Fungicides							
Azoxystrobin	C ₂₂ H ₁₇ N ₃ O ₅	+	404>372	19	404>344	27	9.857
Boscalid	C ₁₈ H ₁₂ Cl ₂ N ₂ O	+	343>307	20	343>272	32	10.122
Prothioconazole	C ₁₄ H ₁₅ Cl ₂ N ₃ OS	+	346>328	8	344>326	8	10.701
Internal standards							
Thiamethoxam-d3		+	295>214	20			3.428
Clothianidin-d3		+	253>172	20			4.012
Imidacloprid-d4		+	260>213	20			4.313
Acetamiprid-d3		+	226>126	30			4.594
Thiacloprid-d4		+	257.1>125.8	30			5.542
Malathion-(diethyl-d10)		+	341.2>132.2	20			10.523
MCPA-(methyl-d3)		-	202>144	20			8.269

Table B4. Performance of the analytical method for each pesticide in pollen and nectar.

	Nectar					Pollen				
	LOD (ppb)	LOQ (ppb)	Linearity (r^2)	Mean recovery (%)	RSD (%)	LOD (ppb)	LOQ (ppb)	Linearity (r^2)	Mean recovery (%)	RSD (%)
Neonicotinoids										
Thiamethoxam	0.03	0.09	0.9980	107.6	1.0	0.03	0.10	0.9993	85.8	3.2
Clothianidin	0.03	0.09	0.9984	123.0	1.0	0.04	0.13	0.9983	91.4	1.4
Imidacloprid	0.05	0.18	0.9984	111.7	0.6	0.09	0.29	0.9989	89.1	3.2
Acetamiprid	0.01	0.03	0.9985	118.7	0.4	0.04	0.12	0.9988	82.9	3.3
Thiacloprid	0.02	0.07	0.9982	121.8	2.0	0.02	0.07	0.9994	117.0	2.0
Herbicide										
Fluroxypyr	0.37	1.25	0.9968	110.9	3.2	0.38	1.28	0.9997	90.3	12.9
Fungicides										
Azoxystrobin	0.004	0.01	0.9941	120.8	1.3	0.01	0.03	0.9941	114.9	2.0
Boscalid	0.01	0.04	0.9987	118.2	4.8	0.13	0.43	0.9997	102.3	2.9
Prothioconazole	0.35	1.17	0.9906	90.0	8.1	0.60	2.00	0.9990	75.9	4.5

Table B5. Results of the Mann-Whitney U tests on the compounds detected in the various matrices.

Compound	Matrix	U	p value
Azoxystrobin	OSR nectar vs OSR pollen	8	0.009
	OSR pollen vs BAB pollen	15	0.072
	OSR pollen vs BAB nectar	0	<0.001
	OSR nectar vs BAB nectar	20	0.200
	OSR nectar vs BAB pollen	20	0.217
	BAB nectar vs BAB pollen	0	<0.001
Boscalid	OSR nectar vs OSR pollen	11.5	0.01
	OSR pollen vs BAB pollen	15	0.041
	OSR pollen vs BAB nectar	8	0.001
	OSR nectar vs BAB nectar	28	>0.999
	OSR nectar vs BAB pollen	15	0.04
	BAB nectar vs BAB pollen	16	0.059
Clothianidin	OSR nectar vs OSR pollen	6	0.714
	OSR pollen vs BAB pollen	6	> 0.999
	OSR pollen vs BAB nectar	7	> 0.999
	OSR nectar vs BAB nectar	7	> 0.999
	OSR nectar vs BAB pollen	4	0.429
	BAB nectar vs BAB pollen	4	0.429
Prothioconazole	OSR nectar vs OSR pollen	2	0.400

Table B6. The acute and chronic Risk Quotients of the maximum concentrations of the five compounds detected in plant pollen and nectar.

			Risk Quotient (RQ)							
			Maximum residues ($\mu\text{g}/\text{kg}$) ^c		Mean residues ($\mu\text{g}/\text{kg}$) ^d		Acute		Chronic	
			Pollen	Nectar	Pollen	Nectar	Foragers	In-hive	Foragers	In-hive
	Oral honey bee LD50 ($\mu\text{g}/\text{bee}$) ^a	Chronic NOED ($\mu\text{g}/\text{bee}/\text{day}$) ^b								
Azoxystrobin	>25	2.5	31.72	2.67	3.35	1.28	0.000031	0.000027	0.000150	0.000085
Boscalid	>166	16.6	1019.82	11.02	133.9	0.6	0.000019	0.000068	0.000011	0.000082
Clothianidin	0.004	0.00072	0.02	0.95	0.07	0.27	0.069350	0.033298	0.109500	0.053433
Glyphosate	104	10.4	17.5	205.7	17.5	127.5	0.000578	0.000279	0.003580	0.001733
Prothioconazole	>71	7.1	95.69	20.87	49.92	19.99	0.000086	0.000054	0.000822	0.000462
Thiacloprid	17.32	1.73	4.43	0.00	4.43	0.00	0.000000	0.000002	0.000000	0.000025

^a The values represent adult honey bee worst case from 24, 48 and 72 h oral acute LD₅₀ (see references PPDB, 2022).

^b The values represent adult honey bee 10-day chronic studies (see references US EPA OPP, 2017).

^c The maximum concentration of residues was used for the acute risk quotient calculation.

^d The mean concentration of residues was used for the chronic risk quotient calculation.

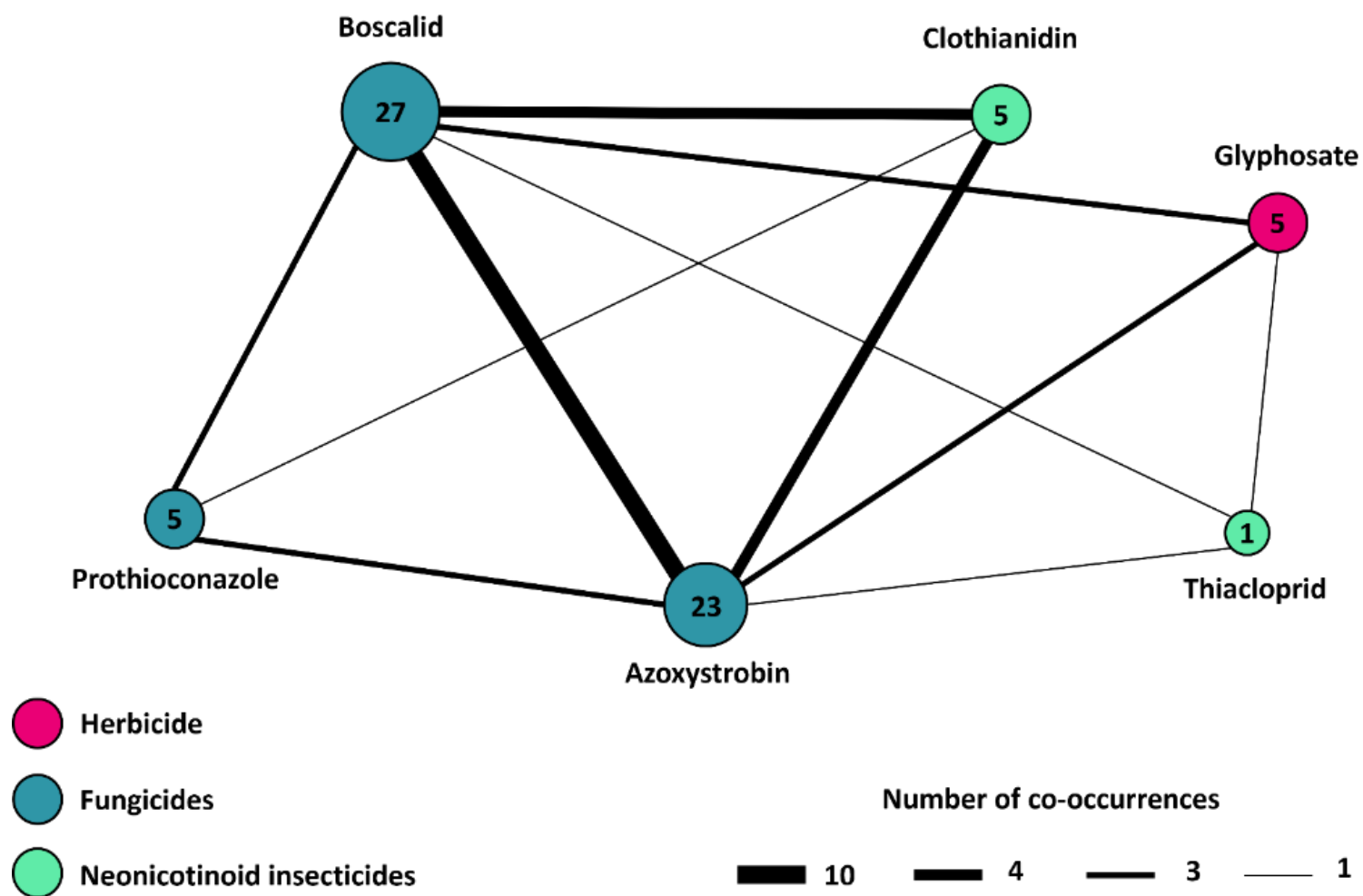


Figure B1. The combinations of the detected compounds that co-occurred in each of the ten *Brassica napus* (oilseed rape) fields. The number inside the bubbles represents the number of total detections of each compound.

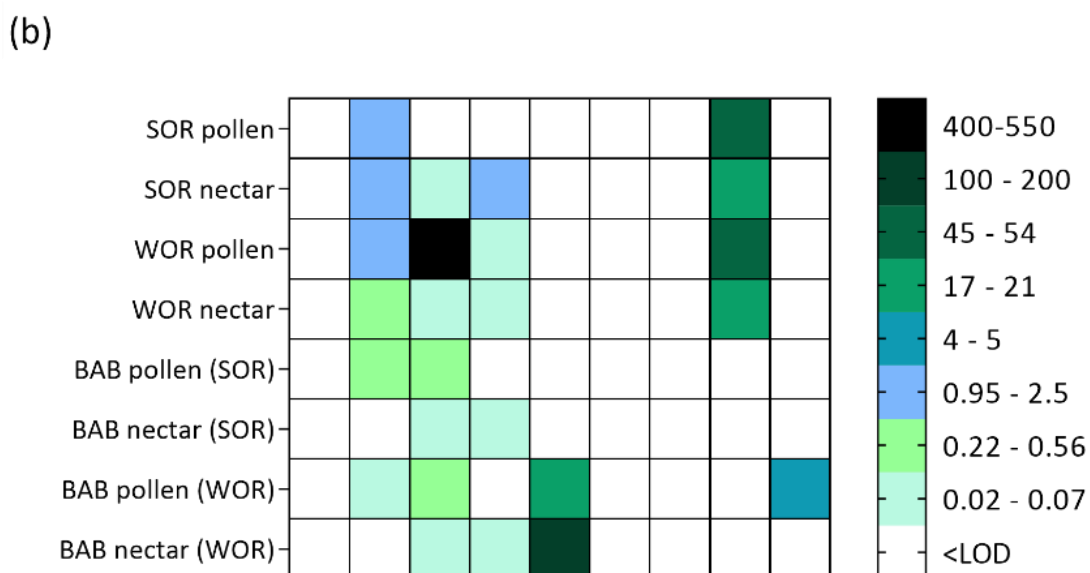
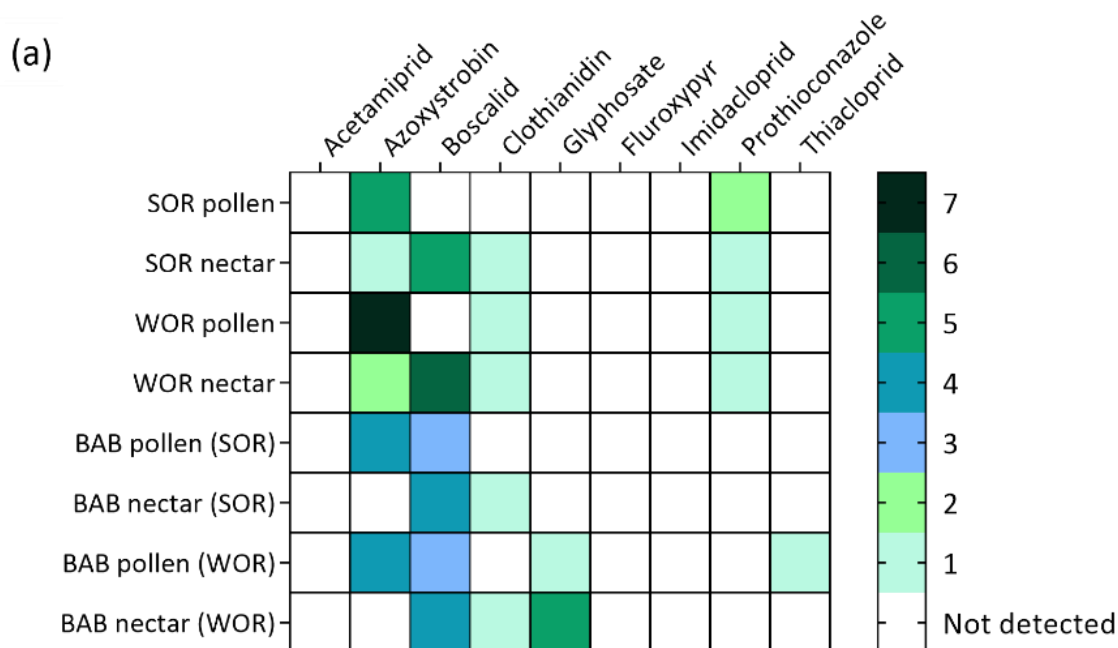


Figure B2. Heatmaps showing (a) the number of detections and (b) the median values of the concentrations of the nine active substances in spring oilseed rape (SOR) pollen and nectar, winter oilseed rape (WOR) pollen and nectar, blackberry (BAB) pollen and nectar. The units of (b) are $\mu\text{g/kg}$.

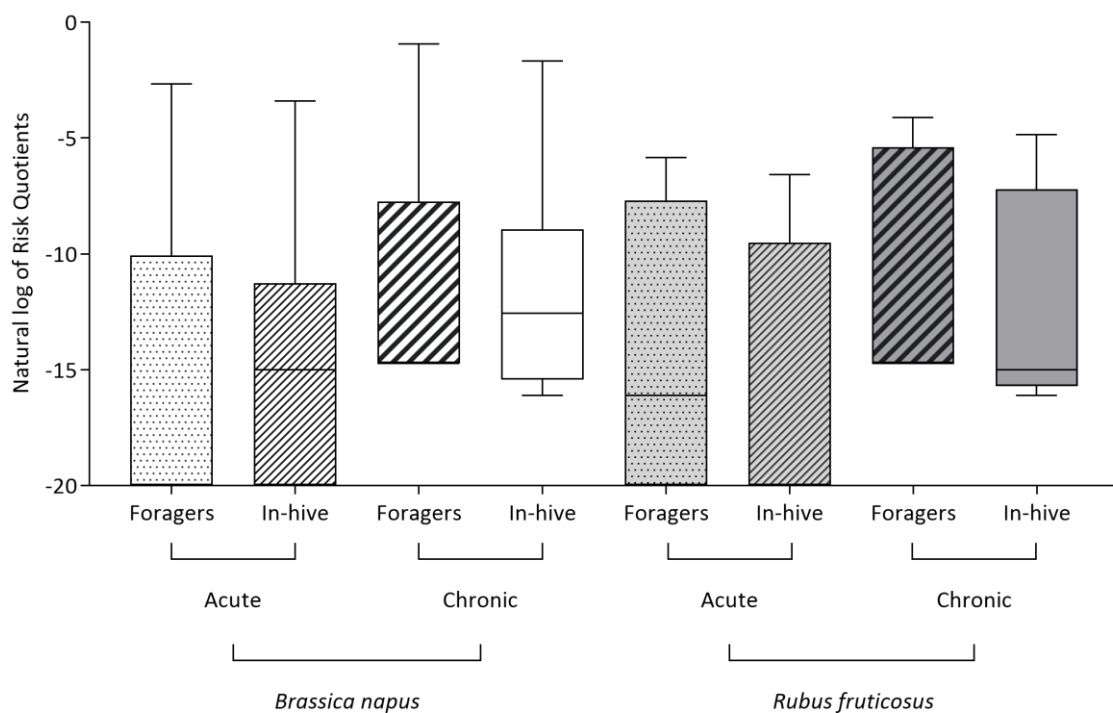


Figure B3. The acute and chronic Risk Quotients of the pesticide residues detected in *Brassica napus* (oilseed rape) and *Rubus fruticosus* (blackberry) plants.

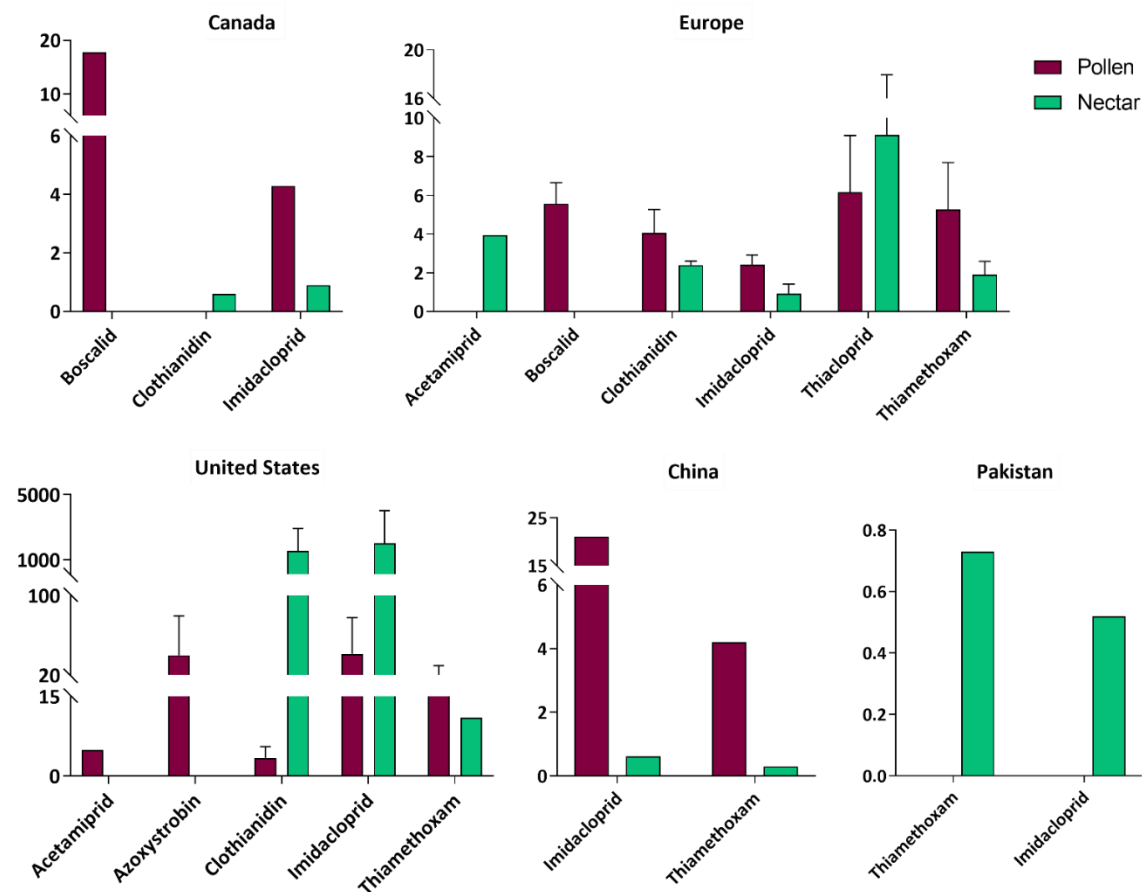


Figure B4. Worldwide detections in plant pollen and nectar of the compounds evaluated in the present study. The countries shaped in grey show the origin of the studies represented as a total for each continent (Asia, North America - Canada and United States, and Europe) in each bar chart. On the y axis we observe the compounds detected in plant pollen and nectar respectively and on the x axis their mean concentrations with standard error bars detected in µg/kg. No residues were recorded for the compounds glyphosate, AMPA, prothioconazole and fluroxypyr. Citations can be found below.

Figure B4. Citations.

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Appendix C - Supplementary data for Chapter 4

Table C1. This table shows all the pesticide applications and the related information on the 12 evaluated fields during the year of study (2019-2020).

Field	Date	Method	BBCH stage	Type	Product	Systemic	Active ingredient	Concentration	Application rate	Formulation type
1	8/Jun/20	Spray	GS63	Fungicide	Az-tec®	Yes	Azoxystrobin	250 g/L	0.25 L/ha	Suspension concentrate
				Fungicide	Fezan®	Yes	Tebuconazole	251 g/L	0.33 L/ha	Emulsifiable concentrate
2	22/Apr/20	Spray	GS20	Herbicide	Stomp®	Yes	Pendimethalin	455 g/L	2.8 L/ha	Capsule suspension
	15/May/20	Spray	GS39	Herbicide	Stratos®	Yes	Cycloxydim	100 g/L	1.5 L/ha	Emulsifiable concentrate
3	16/May/20	Spray	GS20	Herbicide	Stratos®	Yes	Cycloxydim	100 g/L	1.5 L/ha	Emulsifiable concentrate
				Adjuvant	Kantor®		Alkoxylated triglycerides	790 g/L	0.2 L/ha	Emulsifiable concentrate
4	24/Jan/20	Spray	-	Herbicide	Mizr®	Yes	Glyphosate	360 g/L	2.5 L/ha	Soluble concentrate
	1/Apr/20	Spreader	GS10-18	Molluscicide	Axcela®		Metaldehyde	3% w/w	5 kg/ha	Pellets
	11/Apr/20	Spreader	GS10-18	Molluscicide	Axcela®		Metaldehyde	3% w/w	7 kg/ha	Pellets
	8/May/20	Spray	GS29	Insecticide	Ambush®	No	Cypermethrin	100 g/L	0.25 L/ha	Emulsifiable concentrate
	14/May/20	Spray	GS32	Herbicide	Falcon®	Yes	Propaquizafop	100 g/L	0.75 L/ha	Emulsifiable concentrate
5	3/May/20	Spray	GS50	Fungicide	Rover®	No	Chlorothalonil	500 g/L	1 L/ha	Suspension concentrate
				Herbicide	Falcon®	Yes	Propaquizafop	100 g/L	0.75 L/ha	Emulsifiable concentrate
6	27/Mar/20	Spray	GS00-09	Herbicide	Stallion®	Yes	Pendimethalin	333 g/L	3 L/ha	Capsule suspension
							Clomazone	30 g/L		

	28/Apr/20	Spray	GS20	Herbicide	Basagran®	Medium	Bentazone	87 % w/w	0.8 kg/ha	Soluble granule
	13/Jun/20	Spray	GS63	Fungicide	Signum®	Yes	Boscalid Pyraclostrobin	26.7 % w/w 6.7 % w/w	0.75 kg/ha	Dispersible granule
7	8/May/20	Spray	GS10-18	Herbicide	Falcon®	Yes	Propaquizafop	100 g/L	0.5 L/ha	Emulsifiable concentrate
	10/Mar/20	Spray	-	Herbicide	Mizr®	Yes	Glyphosate	360 g/L	2.7 L/ha	Soluble concentrate
				Herbicide	Cleranda®	Yes	Imazamox Metazachlor	17.5 g/L 375 g/L	2 L/ha	Suspension concentrate
8	14/May/20	Spray	GS10-18				Fatty acid esters	348.75 g/L		
				Adjuvant	Dash®		Alkoxylated alcohols- phosphate esters	209.25 g/L	1 L/ha	Emulsifiable concentrate
	25/May/20	Spray	GS39	Fungicide	Proline®	Yes	Prothioconazole	250 g/L	0.4 L/ha	Emulsifiable concentrate
				Herbicide	Falcon®	Yes	Propaquizafop	100 g/L	1.5 L/ha	Emulsifiable concentrate
	30/Jun/20	Spray	GS65	Fungicide	Filan®	Yes	Boscalid	500 g/L	0.5 L/ha	Water dispersible granule
9	18/Mar/20	Spray	-	Herbicide	Mizr®	Yes	Glyphosate	360 g/L	2.7 L/ha	Soluble concentrate
	14/May/20	Spray	GS10-18	Herbicide	Falcon®	Yes	Propaquizafop	100 g/L	0.75 l/ha	Emulsifiable concentrate
10	NA									
	25/May/20	Spray	GS10-18	Herbicide	Falcon®	Yes	Propaquizafop	100 g/L	1 L/ha	Emulsifiable concentrate
11				Fungicide	Prolinex®	Yes	Prothioconazole	250 g/L	0.4 L/ha	Emulsifiable concentrate
	24/Jun/20	Spray	GS64	Fungicide	Filan®	Yes	Boscalid	500 g/L	0.5 L/ha	Water dispersible granule
12	18/May/20	Spray	GS10-18	Herbicide	Falcon®	Yes	Propaquizafop	100 g/L	0.5 L/ha	Emulsifiable concentrate

Table C2. Information related to the 12 fields (location, size, crop variety, sowing date, adjacent fields) and information regarding the sampling (date of sampling, mean temperature recorded, plant species sampled).

Field	County	Variety	Sowing	Crop	Adjacent fields	Temperature (°C)	Sampling date	Pollen origin
1	Meath	Boxer	30/Mar/20	Bean	Grass	15	09/Jun/20	Flowers
						21	15/Jun/20	Bumble bee corbiculae Honey bee corbiculae
2	Kildare	Fanfare	23/Mar/20	Bean	Crop	15	07/Jun/20	Flowers
								Bumble bee corbiculae Honey bee corbiculae
3	Kildare	Lynx	01/Apr/20	Bean	Grass	16	08/Jun/20	Flowers
								Bumble bee corbiculae Honey bee corbiculae
4	Kildare	Lynx	21/Mar/20	Bean	Crop	15	08/Jun/20	Flowers
								Bumble bee corbiculae Honey bee corbiculae
5	Laois	Wizard	28/Oct/19	Bean	Grass	17	24/May/20	Flowers
								Bumble bee corbiculae Honey bee corbiculae
6	Laois	Lynx	24/Mar/20	Bean	Crop	16	14/Jun/20	Flowers
								Bumble bee corbiculae

								Honey bee corbiculae
7	Kildare	Lumen	10/Apr/20	Oilseed rape	Crop	15	09/Jun/20	Flowers
								Bumble bee corbiculae
						18	13/Jun/20	Honey bee corbiculae
8	Kildare	Hybrid	09/Apr/20	Oilseed rape	Crop	17	15/Jun/20	Flowers
								Bumble bee corbiculae
								Honey bee corbiculae
						15	30/Jun/20	Flowers
								Bumble bee corbiculae
		Honey bee corbiculae						
9	Laois	Hybrid	09/Apr/20	Oilseed rape	Crop	24	01/Jun/20	Flowers
								Bumble bee corbiculae
								Honey bee corbiculae
10	Kildare	Lumen	03/Apr/20	Oilseed rape	Crop	21	31/May/20	Flowers
								Bumble bee corbiculae
								Honey bee corbiculae
11	Kildare	Hybrid	04/04/2020	Oilseed rape	Grass	13	12/06/2020	Flowers
								Bumble bee corbiculae
								Honey bee corbiculae
						20	25/06/2020	Flowers

								Bumble bee corbiculae
								Honey bee corbiculae
								Flowers
12	Kildare	Lumen	16/04/2020	Oilseed rape	Grass	16	20/06/2020	Bumble bee corbiculae
								Honey bee corbiculae

Table C3. The percentage of plant family or plant species in the honey bee (*Apis mellifera*) and bumble bee (*Bombus* sp.) pollen from foragers on bean and oilseed rape crops.

Field	Sampling date	Crop	Bee species	Plant family/species	Percentage (%)
1	09-Jun	Bean	<i>Apis mellifera</i>	Asteraceae	0.05
				Brassicaceae	0.4
				Ericaceae	0.05
				Fabaceae	0.6
				Rosaceae	0.1
				Viburnaceae	0.15
				<i>Vicia faba</i>	98.65
			<i>Bombus</i> sp.	Apiaceae	0.06
				Asteraceae	0.18
				Brassicaceae	14.31
				Ericaceae	0.03
				Fabaceae	12.93
				Ranunculaceae	1.09
				Rosaceae	15.61
				Solanaceae	14.84
				Viburnaceae	0.31
				<i>Vicia faba</i>	40.64
2	07-Jun	Bean	<i>Apis mellifera</i>	Asteraceae	0.1
				<i>Brassica napus</i>	0.45
				Fabaceae	0.2
				Rosaceae	0.2
				Sapindaceae	0.05
				Viburnaceae	0.15
				<i>Vicia faba</i>	98.85
			<i>Bombus</i> sp.	Asteraceae	0.7
				<i>Brassica napus</i>	8.3
				Fabaceae	20.1
				Papaveraceae	0.1
				Rosaceae	13.5
				Sapindaceae	0.04
				Viburnaceae	0.3

			<i>Vicia faba</i>	57.0
			Violaceae	0.1
			<i>Brassica napus</i>	0.05
		<i>Apis mellifera</i>	Fabaceae	0.1
			<i>Vicia faba</i>	99.85
			Asteraceae	0.3
			Boraginaceae	0.1
			<i>Brassica napus</i>	0.2
3	08-Jun	Bean	Fabaceae	16.9
		<i>Bombus sp.</i>	Rosaceae	0.6
			Solanaceae	19.8
			Viburnaceae	0.1
			<i>Vicia faba</i>	61.9
			Violaceae	0.1
			<i>Brassica napus</i>	0.1
		<i>Apis mellifera</i>	Viburnaceae	0.15
			<i>Vicia faba</i>	99.75
			<i>Brassica napus</i>	4.97
			Caprifoliaceae	0.31
4	08-Jun	Bean	Ericaceae	0.01
		<i>Bombus sp.</i>	Fabaceae	6.47
			Rosaceae	6.07
			Viburnaceae	0.05
			<i>Vicia faba</i>	81.82
			Violaceae	0.30
			Asteraceae	0.05
			Boraginaceae	14.5
		<i>Apis mellifera</i>	<i>Brassica napus</i>	1.45
			Rosaceae	0.15
5	24-May	Bean	<i>Vicia faba</i>	83.85
			Asteraceae	1.3
		<i>Bombus sp.</i>	Boraginaceae	42.45
			<i>Brassica napus</i>	0.2
			Brassicaceae	0.03

6	14-Jun				Fabaceae	20	
					Rosaceae	0.14	
					<i>Vicia faba</i>	35.9	
				Oilseed rape	<i>Apis mellifera</i>	<i>Brassica napus</i>	98.95
						Ericaceae	0.25
						Viburnaceae	0.75
						<i>Vicia faba</i>	0.05
					<i>Bombus</i> sp.	Asteraceae	0.45
						<i>Brassica napus</i>	93.15
						Ericaceae	0.875
						Euphorbiaceae	0.05
						Fabaceae	3.125
						Viburnaceae	0.875
						<i>Vicia faba</i>	1.475
						Bean	<i>Apis mellifera</i>
				Fabaceae	0.15		
				Rosaceae	0.25		
				Viburnaceae	0.05		
				<i>Vicia faba</i>	99.35		
				<i>Bombus</i> sp.	<i>Brassica napus</i>		3.13
					Ericaceae		0.13
					Fabaceae		1.66
					Rosaceae		24.04
					<i>Vicia faba</i>		70.79
					Violaceae	0.25	
7	13-Jun	Oilseed rape	<i>Apis mellifera</i>	Asteraceae	0.25		
				<i>Brassica napus</i>	98.65		
				Brassicaceae	0.55		
				Ranunculaceae	0.1		
				<i>Vicia faba</i>	0.45		
	09-Jun	Oilseed rape	<i>Bombus</i> sp.	Asteraceae	0.025		
				<i>Brassica napus</i>	72.95		
				Brassicaceae	1.875		
				Ericaceae	0.025		
				<i>Vicia faba</i>	25.125		

8	15-Jun	Oilseed rape	<i>Apis mellifera</i>	<i>Brassica napus</i>	99.9
				Fabaceae	0.1
			<i>Bombus</i> sp.	Asteraceae	0.025
				<i>Brassica napus</i>	83.525
				Fabaceae	11
				Poaceae	0.025
				Rosaceae	0.1
				Solanaceae	4.925
				Viburnaceae	0.4
	30-Jun	Oilseed rape	<i>Apis mellifera</i>	<i>Brassica napus</i>	100
				Apiaceae	0.375
			<i>Bombus</i> sp.	<i>Brassica napus</i>	76.125
				Fabaceae	20.8
				Fagaceae	0.275
				Geraniaceae	0.575
				Malvaceae	0.05
				Rosaceae	0.95
9	01-Jun	Oilseed rape	<i>Apis mellifera</i>	Solanaceae	0.1
				Viburnaceae	0.75
				Asteraceae	0.1
				Boraginaceae	0.1
				<i>Brassica napus</i>	99.2
				Fabaceae	0.5
				Ranunculaceae	0.05
				Viburnaceae	0.05
			<i>Bombus</i> sp.	Asteraceae	0.12
				<i>Brassica napus</i>	83.81
				Caprifoliaceae	1.67
				Ericaceae	0.02
				Fabaceae	8.2
				Rosaceae	3.23
				Valerianaceae	0.05
				Viburnaceae	2.87
				Violaceae	0.03
10	31-May	Oilseed rape	<i>Apis mellifera</i>	Asteraceae	0.2

11			Boraginaceae	0.05	
			<i>Brassica napus</i>	99	
			Viburnaceae	0.7	
			<i>Vicia faba</i>	0.05	
			Asteraceae	0.15	
			<i>Brassica napus</i>	96.34	
			<i>Bombus</i> sp.	Brassicaceae	1.81
			Malvaceae	1.075	
			Viburnaceae	0.625	
			20-Jun	Oilseed rape	Asteraceae
	<i>Brassica napus</i>	98.95			
	<i>Apis mellifera</i>	Ericaceae			0.2
	Fabaceae	0.55			
	Viburnaceae	0.25			
	<i>Brassica napus</i>	97.41			
	Brassicaceae	0.02			
	Ericaceae	0.03			
	<i>Bombus</i> sp.	Fabaceae			0.92
	Papaveraceae	0.02			
Rosaceae	1.50				
Solanaceae	0.10				
25-Jun	Oilseed rape	Apiaceae	0.45		
		<i>Brassica napus</i>	94.9		
		<i>Apis mellifera</i>	Brassicaceae	0.4	
		Fabaceae	4		
		Rosaceae	0.25		
		Apiaceae	1.42		
		Asteraceae	0.25		
		<i>Brassica napus</i>	76.42		
		<i>Bombus</i> sp.	Brassicaceae	0.02	
Fabaceae	15.87				
Lamiaceae	0.08				
Rosaceae	5				
Valerianaceae	0.02				

			Viburnaceae	0.92
			<i>Brassica napus</i>	99.25
		<i>Apis mellifera</i>	Fabaceae	0.1
			Viburnaceae	0.55
			<i>Vicia faba</i>	0.1
			Apiaceae	0.6
			Asteraceae	0.65
12	20-Jun	Oilseed rape	<i>Brassica napus</i>	76.55
		<i>Bombus</i> sp.	Fabaceae	6.05
			Papaveraceae	0.2
			Rosaceae	15.55
			Viburnaceae	0.35
			<i>Vicia faba</i>	0.05

Table C4. Results of the Mann-Whitney U tests on the pesticide residues detected among the different pollen types.

Pollen type	U	p value
OSR Honey bee vs OSR Crop	94	0.829
OSR Bumble bee vs OSR Crop	143.5	0.565
OSR Bumble bee vs OSR Honey bee	92	0.764
OSR Crop vs BEA crop	29	0.589
BEA Honey bee vs OSR Crop	31.5	0.712
BEA Bumble bee vs OSR Crop	40	0.731
OSR Honey bee vs BEA Crop	19	0.756
OSR Honey bee vs BEA Honey bee	16.5	0.51
BEA Bumble bee vs OSR Honey bee	26	0.911
OSR Bumble bee vs BEA Crop	30	0.642
BEA Honey bee vs OSR Bumble bee	23.5	0.303
OSR Bumble bee vs BEA Bumble bee	42	0.856
BEA Honey bee vs BEA Crop	4	0.342
BEA Bumble bee vs BEA Crop	8	0.706
BEA Honey bee vs BEA Bumble bee	6	0.381

Table C5. Results of the Mann-Whitney U tests on the on the compounds detected in the various pollen types.

Compound	Pollen type	U	p value
Azoxystrobin	Honey bee vs Bumble bee	2	0.142
	Crop vs Honey bee	14	> 0.999
	Crop vs Bumble bee	5.5	0.034
Boscalid	Honey bee vs Bumble bee	13	0.792
	Crop vs Honey bee	33.5	0.501
	Crop vs Bumble bee	20	0.181
Thiamethoxam	Honey bee vs Bumble bee	15.5	0.858

Table C6. Mean pesticide residues detected in the three different pollen types (plant, honey bee and bumble bee corbiculae) in the present study and the worldwide literature.

Mean pesticide residues in pollen (µg/kg)						
	Present study			Worldwide studies		
	Plant	Honey bee	Bumble bee	Plant	Honey bee	Bumble bee
Acetamiprid	-	-	7.7 ± 10.9	4.9	19.7 ± 34.6	-
Azoxystrobin	0.2 ± 0.36	0.02 ± 0.0	1.05 ± 0.8	40.1 ± 55.8	59.9 ± 130.8	44.4
Boscalid	1574.0 ± 3158.9	1209.4 ± 2189.5	650.7 ± 869.2	7.3 ± 5.2	2185.5 ± 6131.9	2534.6 ± 3684.8
Clothianidin	-	-	5.5 ± 7.7	3.8 ± 3.6	25.8 ± 94.3	0.9 ± 0.7
Imidacloprid	-	-	19.2	18.5 ± 44.1	12.3 ± 19.2	0.7
Thiacloprid	-	-	22.2 ± 31.3	6.2 ± 7.2	83.3 ± 169.6	2.9
Thiamethoxam	-	3.2 ± 5.4	2.9 ± 6.5	9.1 ± 19.9	8.8 ± 16.1	9.0 ± 12.7

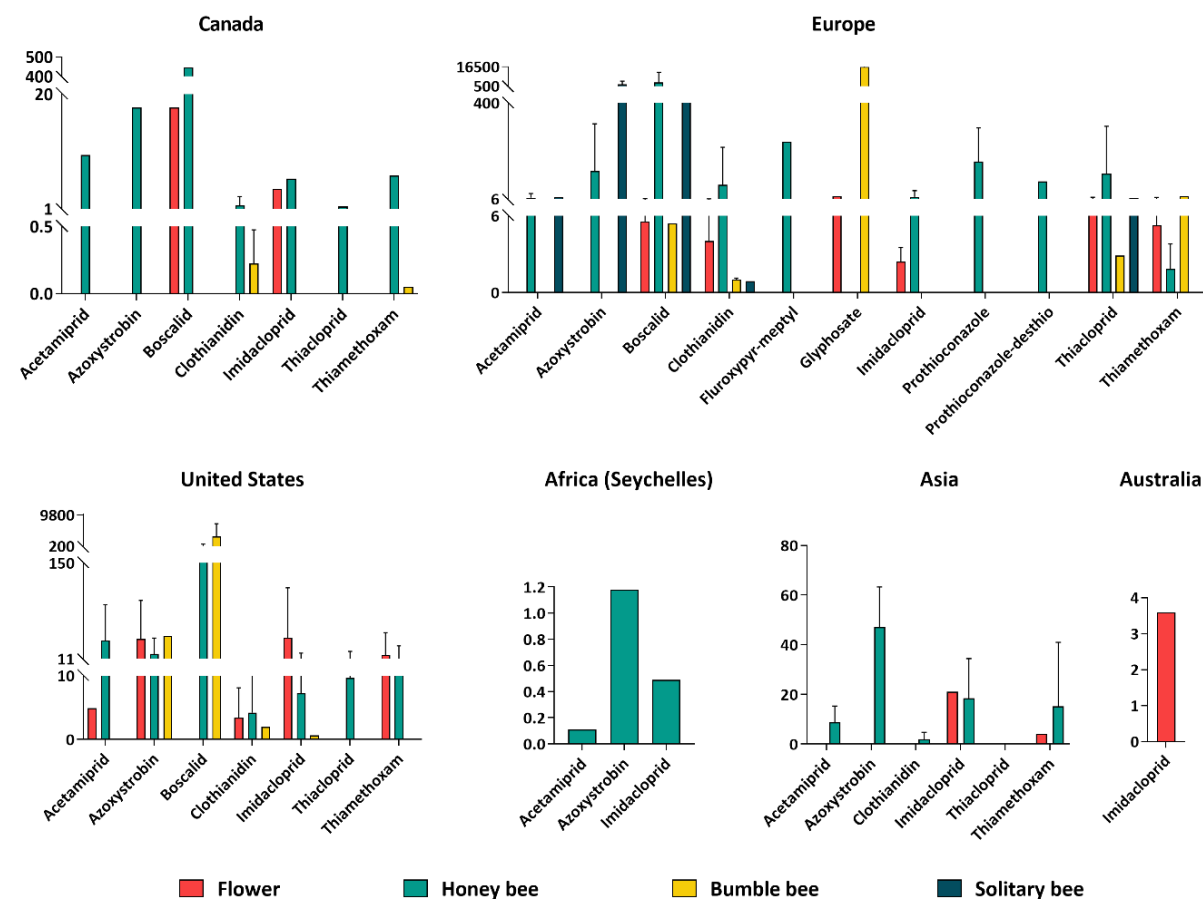


Figure C1. Worldwide detections in flower and bee collected pollen of the compounds evaluated in the present study. The countries shaped in grey show the origin of the studies represented as a total for each continent (Africa - Seychelles, Asia, Australia, North America - Canada and United States, and Europe) in each bar chart. On the y axis we observe the compounds detected and on the x axis their mean concentrations with standard error bars detected in $\mu\text{g/kg}$. The citations of the studies can be found below.

Figure C1. Citations.

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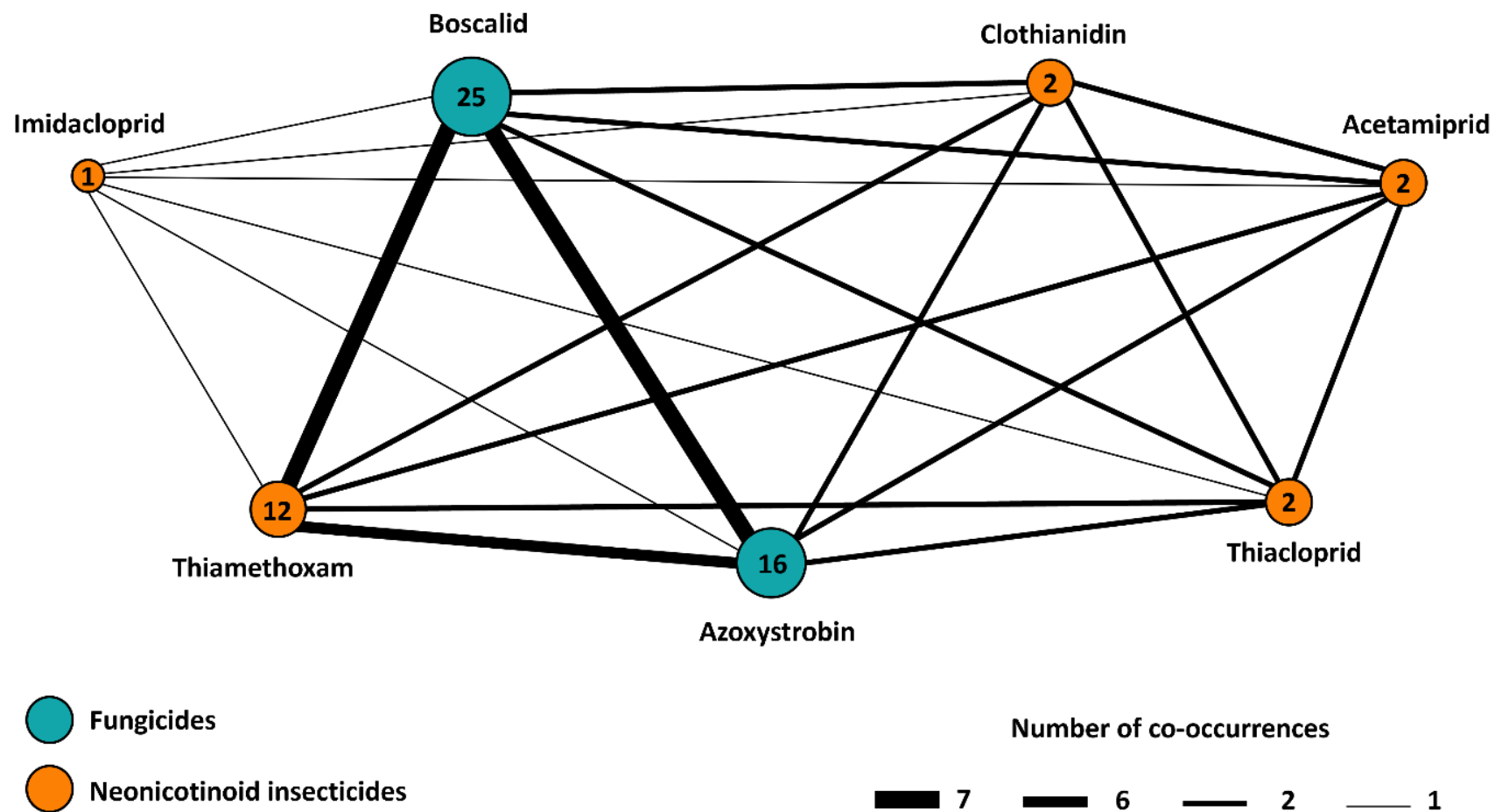
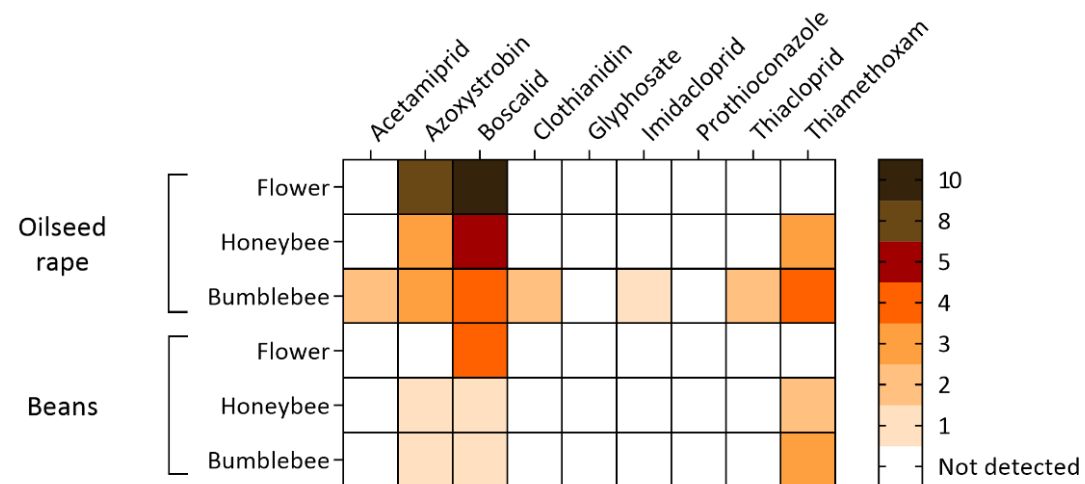


Figure C2. Co-occurrences of the seven detected compounds in the evaluated fields. The number of each circle represents the number of detections for each compound.

(a)



(b)

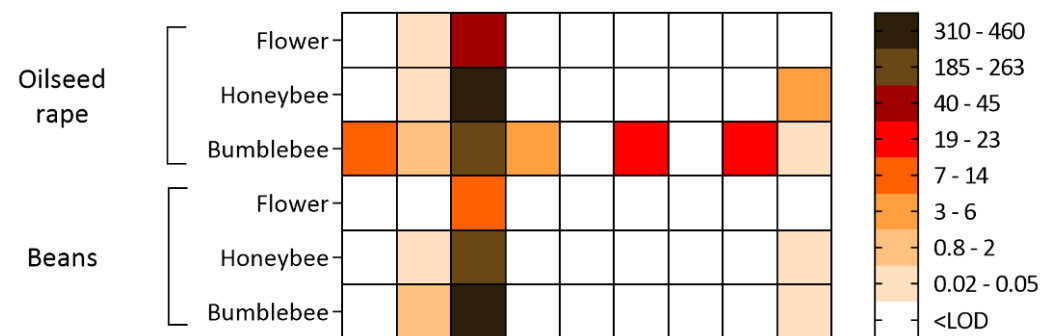


Figure C3. Heatmaps showing (a) the number of detections and (b) the median values of the concentrations of the active substances in flower, honey bee and bumble bee collected pollen.

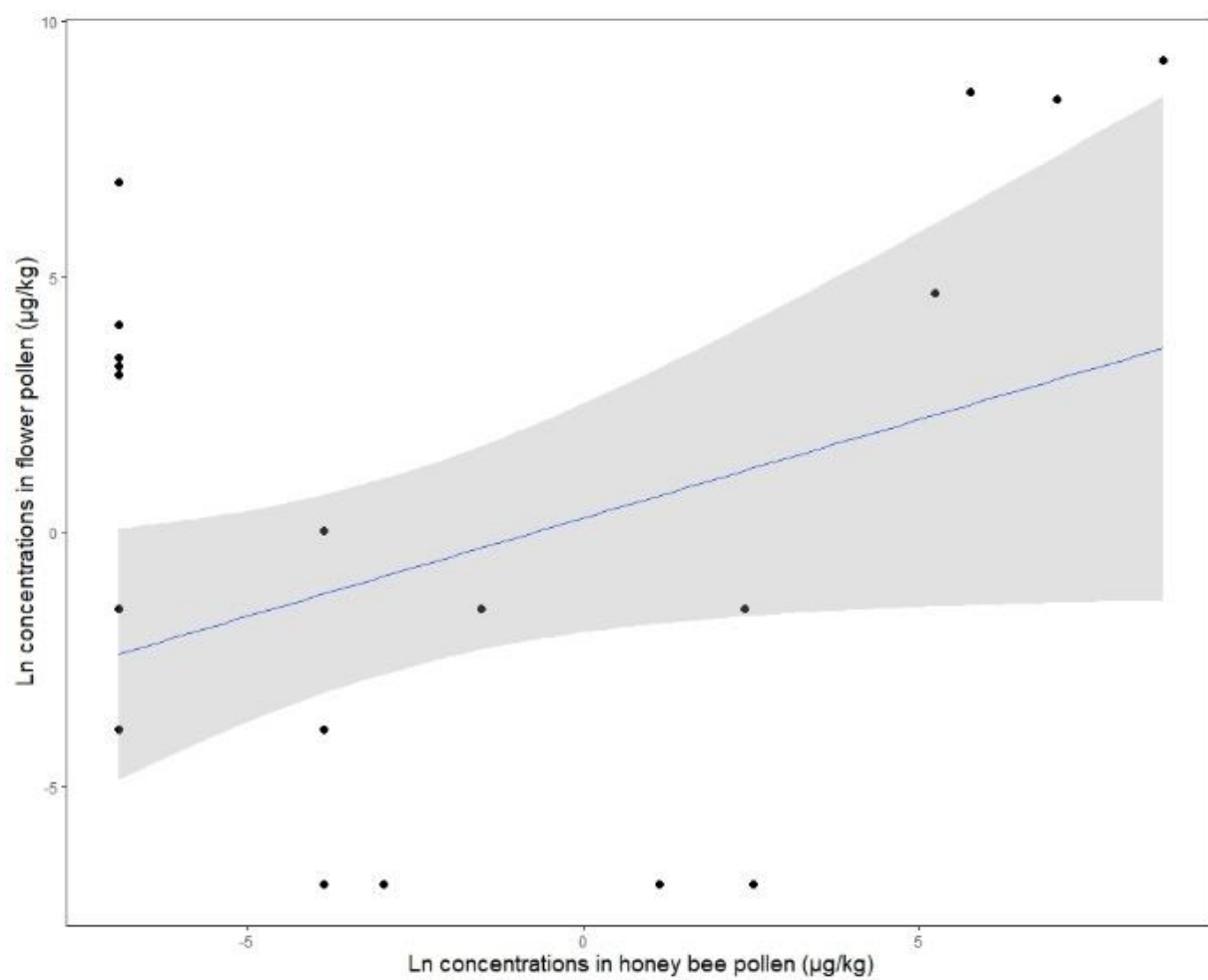
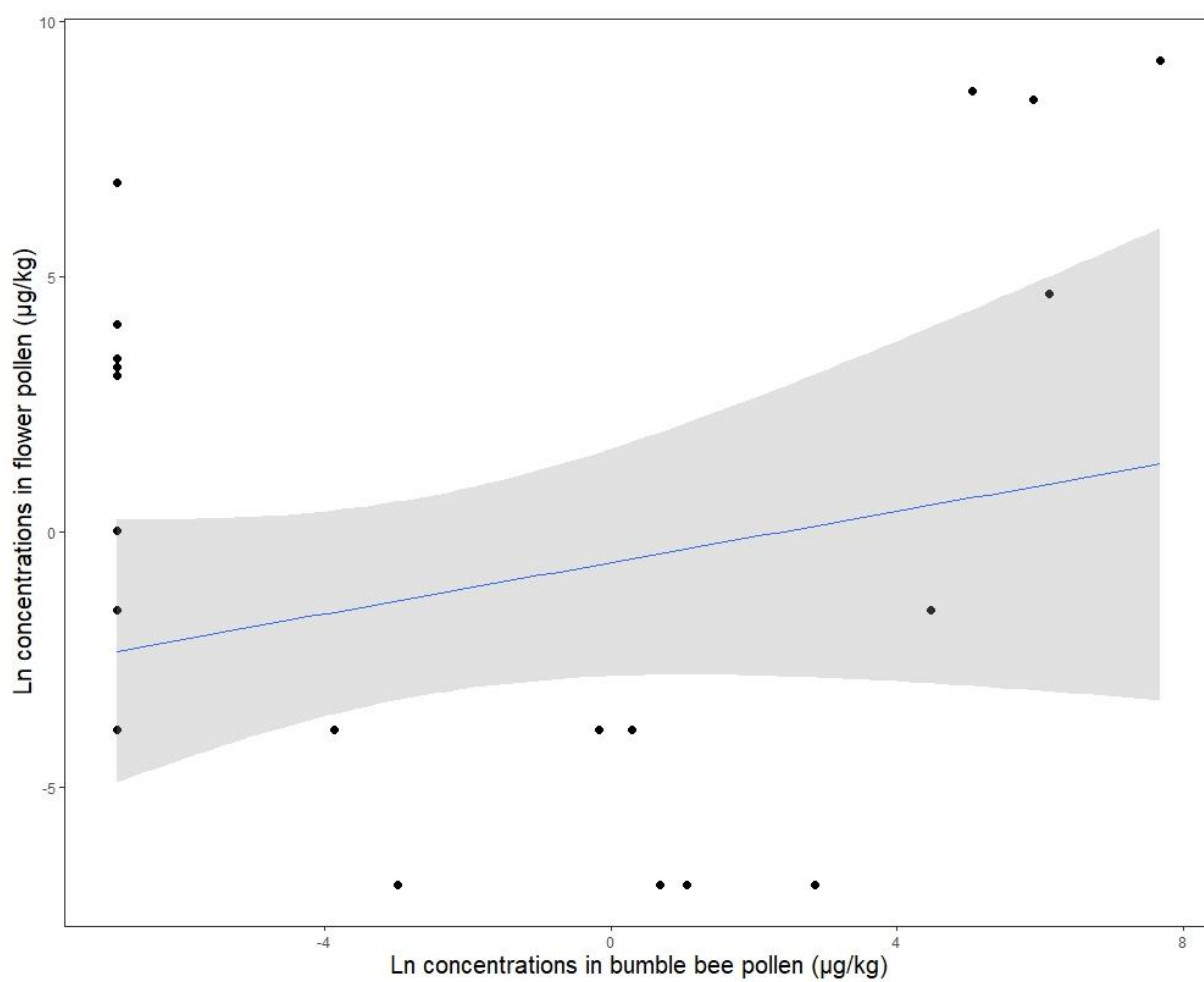


Figure C4. Positive correlation of the azoxystrobin, boscalid and thiamethoxam residues detected in flower and honey bee pollen.



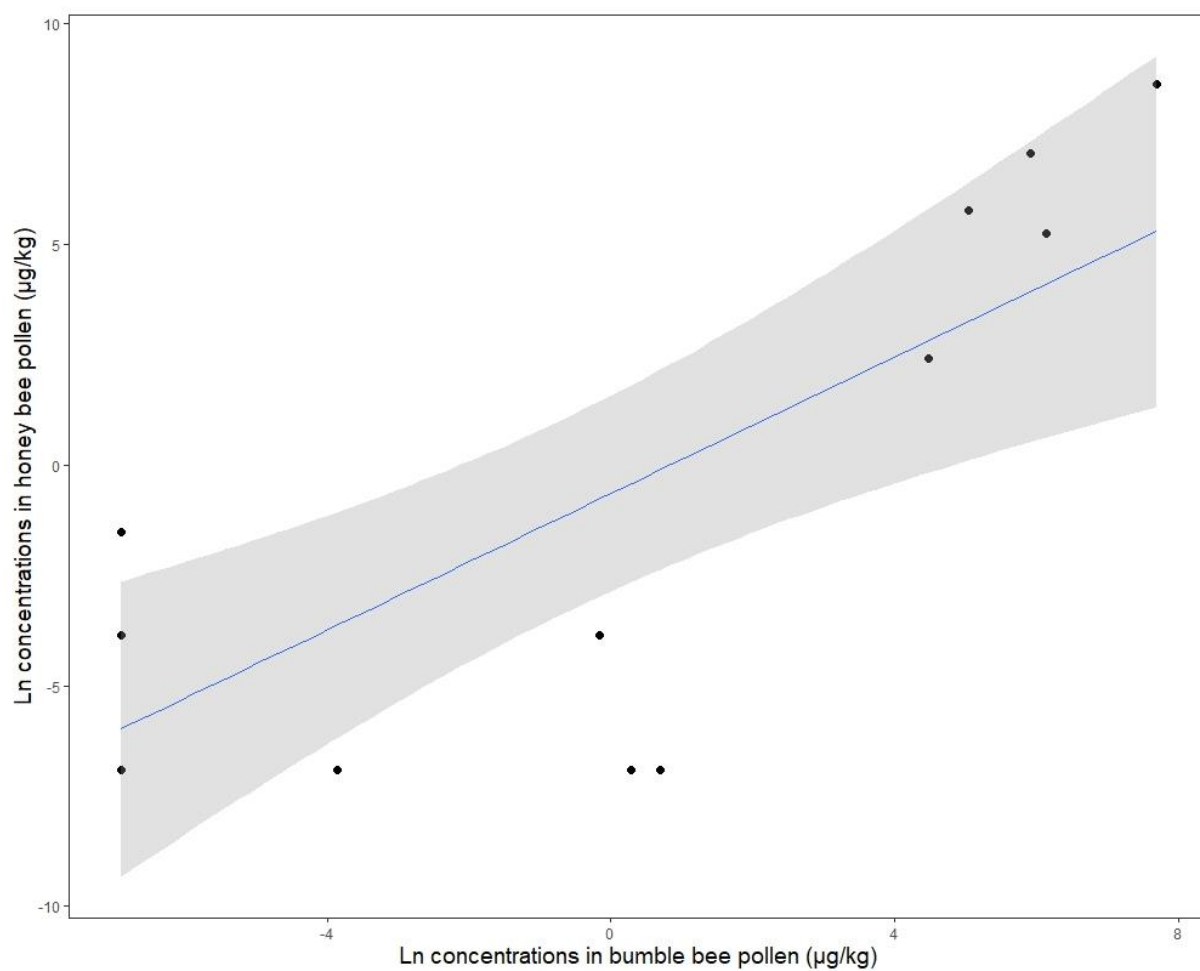


Figure C6. Positive correlation of fungicide residues detected in honey bee and bumble bee pollen.

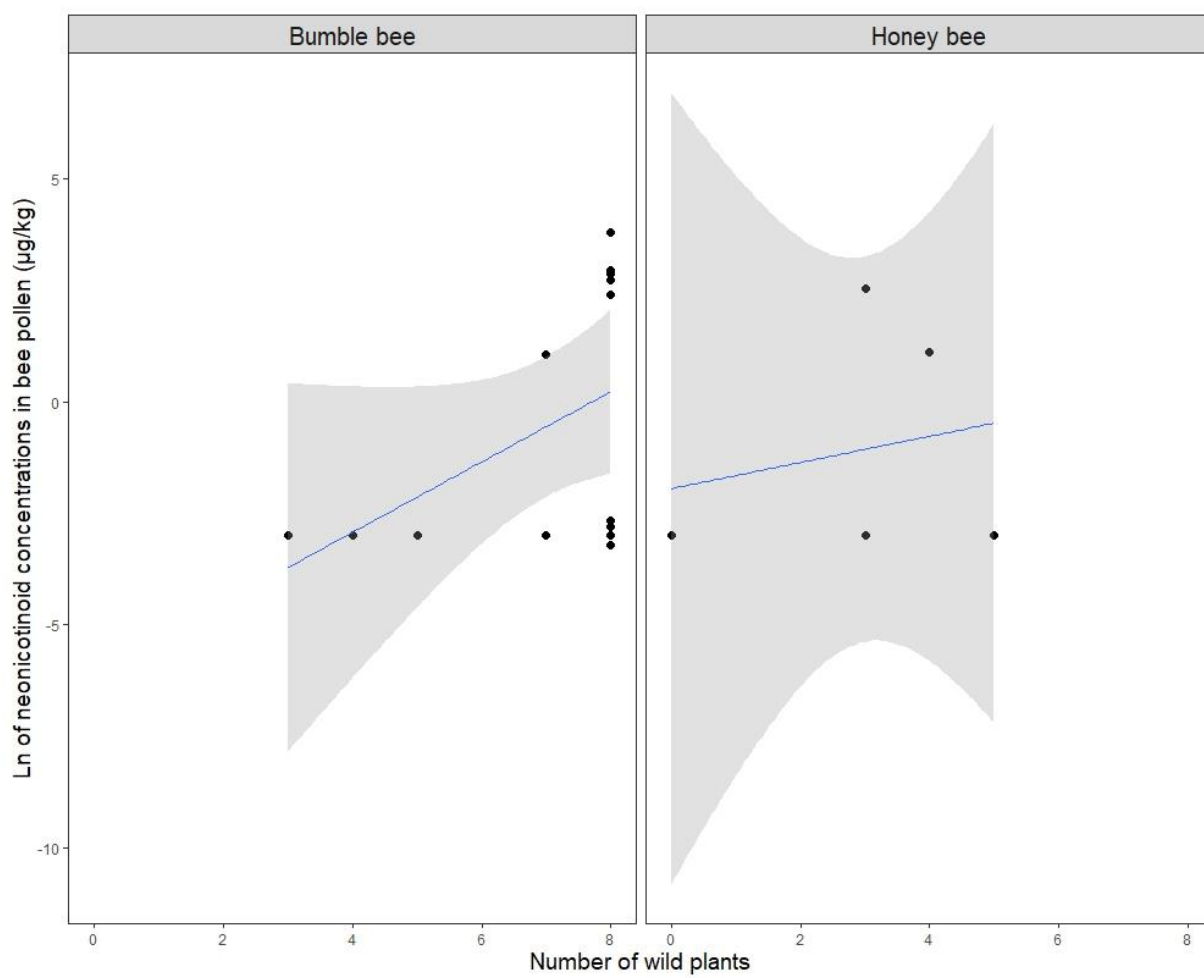


Figure C7. Positive correlation of insecticide residues and number of wild plants detected in bumble bee pollen.

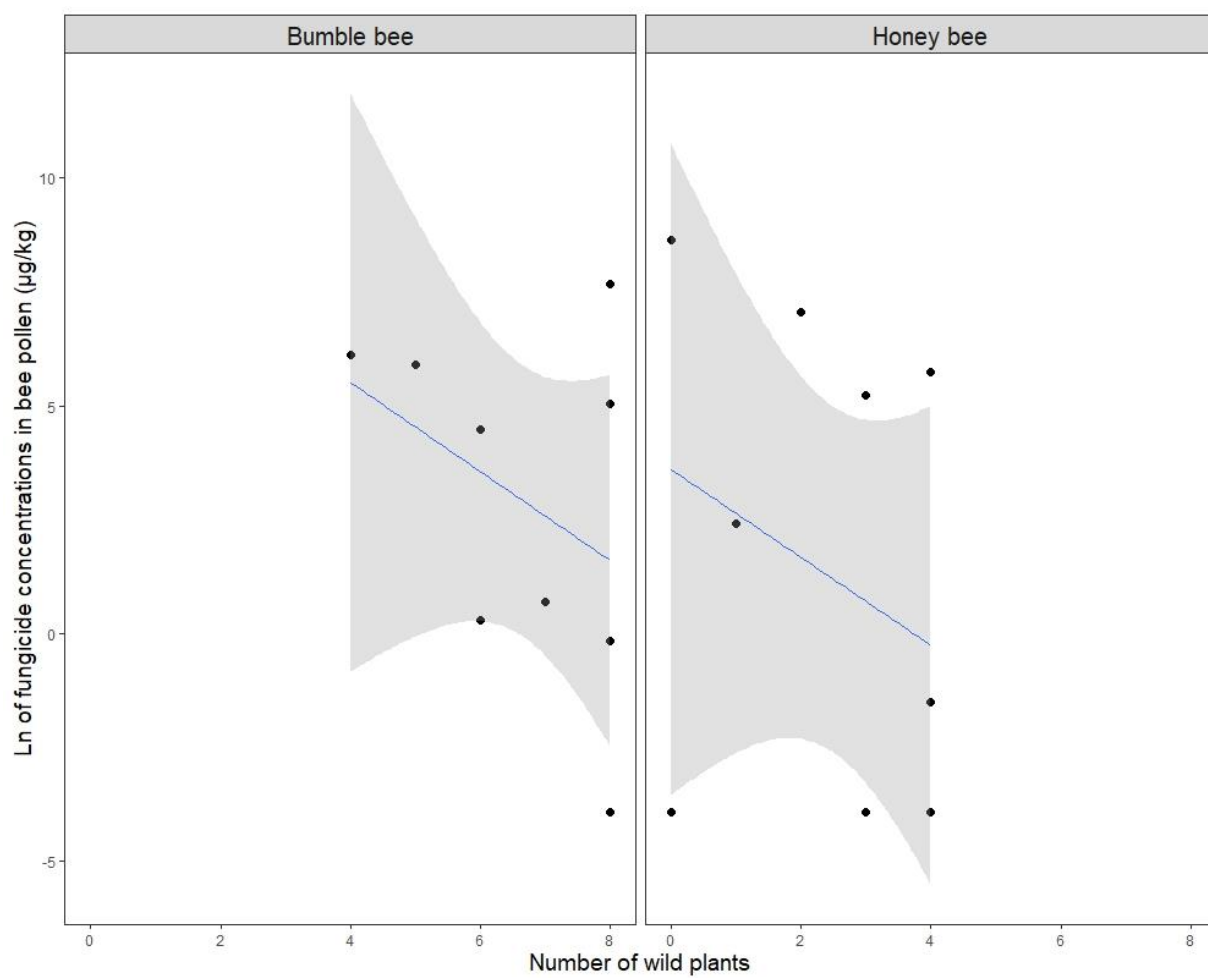


Figure C8. Negative correlation of fungicide residues and number of wild plants detected in bee collected pollen.

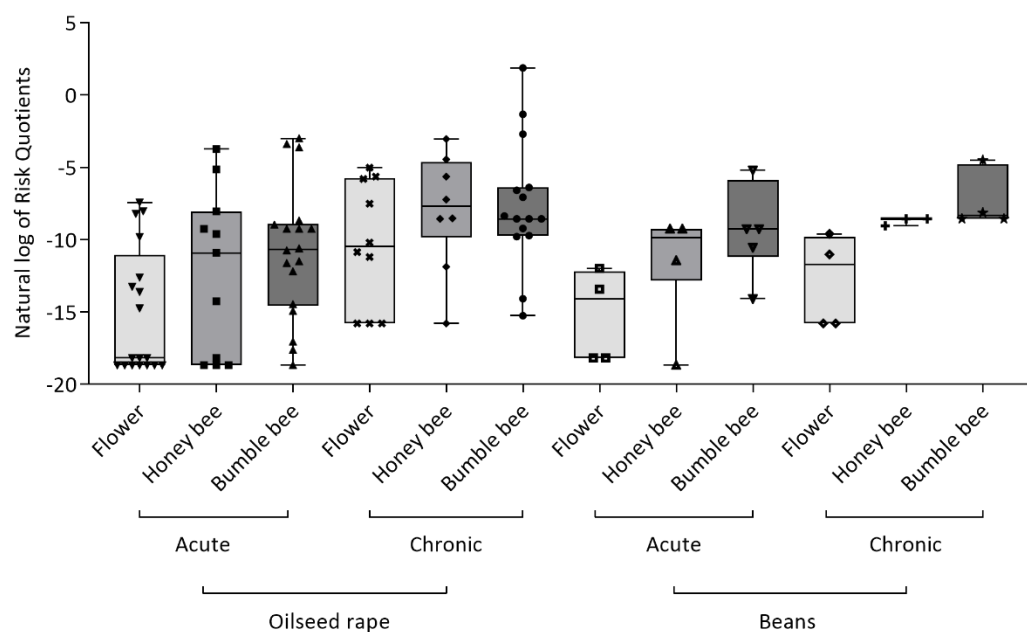


Figure C9. The acute and chronic Risk Quotients of the pesticide residues detected in oilseed rape and bean fields.

Appendix D - Supplementary data for Chapter 5

Table D1. Information on glyphosate-based product applications on the seven sampled oilseed rape fields.

Field	Date	Method	Product	Concentration	Application rate	Formulation type
1	11-Jul-19	Spray	Roundup Gold 450®	450 g/L	1.5 L/ha	Concentrate
2	10-Jul-19	Spray	Roundup PowerMax®	720 g/kg	2 kg/ha	Granule
3	01-Oct-18	Spray	Roundup Gold 450®	450 g/L	1.5 L/ha	Concentrate
4	16-Jul-19	Spray	RoundUp PowerMax®	720 g/kg	1.7 kg/ha	Granule
5	09-Mar-19	Spray	Mizr XL®	360 g/L	2.5 L/ha	Concentrate
6	10-Mar-20	Spray	Mizr XL®	360 g/L	2.7 L/ha	Concentrate
7	18-Mar-20	Spray	Mizr XL®	360 g/L	2.7 L/ha	Concentrate

Table D2. Information on the characteristics of the seven fields, the temperature, the sampling date, and the plant species sampled.

Field	County	Crop variety	Crop Type	Sowing date	Adjacent fields	Temp (°C)	Sampling date	Matrix evaluated
1	Wicklow	DK exception	Winter	27-Aug-18	Grass	4	31-Mar-19	OSR nectar
						17	13-Jul-19	BAB nectar
2	Wicklow	Phoenix CL	Winter	24-Aug-18	Crop	5	10-Apr-19	OSR nectar
						17	13-Jul-19	BAB pollen & nectar
						19	15-Jul-19	BAB nectar
3	Wicklow	DK exception	Winter	21-Aug-18	Crop	8	06-Apr-19	OSR pollen & nectar
						15	06-Jul-19	BAB pollen & nectar
4	Kildare	DK exception	Winter	30-Aug-18	Grass	7	11-Apr-19	OSR nectar
						12	11-May-19	OSR nectar
						18	18-Jul-19	BAB pollen & nectar
						20	23-Jul-19	BAB nectar
5	Kildare	Hybrid	Spring	06-Apr-19	Crop	20	04-Jul-19	OSR pollen & nectar
						20	09-Jul-19	BAB pollen & nectar
6	Kildare	Hybrid	Spring	09-Apr-19	Crop	17	15-Jun-20	OSR pollen
								Honey bee pollen
								Bumble bee pollen
7	Laois	Hybrid	Spring	09-Apr-19	Crop	24	01-Jun-20	OSR pollen
								Honey bee pollen
								Bumble bee pollen

Table D3. The compound-specific LC-MS/MS retention times (*Rt*), quantifying transition ions (*Q*) and qualifier transition ions (*q*) for glyphosate, AMPA and their internal standards.

Compound	ESI mode	Q (m/z)	CE	q (m/z)	CE	Rt (min)
Glyphosate	(-)	168>150	8	168>63	32	20.086
AMPA	(-)	110>79	36	110>63	20	6.492
Internal standards						
Glyphosate ¹⁵ N	(-)	171>63	24			20.018
AMPA ¹⁵ N	(-)	112>63	20			6.490

Table D4. The parameters evaluated for the nectar and pollen method validation.

	Nectar					Pollen				
	LOD (ppb)	LOQ (ppb)	Linearity (r ²)	Mean recovery (%)	RSD (%)	LOD (ppb)	LOQ (ppb)	Linearity (r ²)	Mean recovery (%)	RSD (%)
Glyphosate	8.0	25.0	0.999	101.9	5.7	12.0	35.0	0.997	107.0	6.1
AMPA	8.0	25.0	0.999	98.1	6.6	12.0	35.0	0.999	99.4	4.7