Acute and chronic effects of agrochemicals, and their synergistic interactions, on bees

Deliverable D3.3

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Pan-european assessment, monitoring, and mitigation of stressors on the health of bees

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Summary

Inter-individual differences in pesticide sensitivity may trigger variability in the risk posed by pesticides. Therefore, to better inform pesticide risk assessment for bees, we studied the variability of responses to several pesticides based on endogenous (developmental stage, genetic background, caste) and exogenous factors (pesticide co-exposure). We mainly investigated the toxicity of the insecticide sulfoxaflor, the fungicide azoxystrobin and the herbicide glyphosate. We first used LD_{50} tests to determine the acute oral and contact toxicity of these pesticides across the different bee species, developmental stages (larva vs adult in honey bees), castes (honey bee and bumble bee workers, queens and drones), and genetic backgrounds (honey bee subspecies). We then considered the risks posed by chronic and sublethal exposures to pesticides by implementing behavioural and reproductive endpoints in the screening of pesticide toxicity.

Data showed that azoxystrobin and glyphosate under the test conditions were mildly toxic to bees. However, a large variability in bee sensitivity to sulfoxaflor was found, especially across species and individuals of different castes or sex. This variability is therefore important to consider for increasing the safety margin of the risk posed by insecticides in bees. Several effects induced by sublethal concentrations or doses of pesticides are also described, such as the occurrence of a Non-Monotonic Dose-Response (NMDR) and delayed effects in honey bees, impairment of reproductive performances in bumble bees, and a decreased longevity of *Osmia* adult females (although no effects were found on larval development). Finally, an interaction between pesticides was found when exposure was by contact, but not under oral exposure. In conclusion, the range of effects described here provides very useful insights for better understanding the toxicity of pesticides and therefore the risks they might pose to bees.

1. Introduction

As part of the overall risk assessment procedure, test guidelines require toxicological data on honey bees (*Apis mellifera*). The effects of pesticides are assessed by standard regulatory tests, in a tiered approach. At a low tier, laboratory tests on active substances or formulated products are used on individual bees. This is the first mandatory step that includes an acute toxicity test after oral or contact exposure in adults and larvae, and a chronic toxicity test on adults over 10 days. For acute toxicity tests, the LD50 is determined. Then, if a risk is identified as a result of this first step, supplementary tests are required at a higher tier (semi-field and field tests).

Until very recently, honey bees were the only insect pollinator used for pesticide risk assessment and were considered a surrogate species for non-*Apis* bees. However, bees from the genus *Osmia* and *Bombus* have been recently proposed as additional test surrogates to honey bees, as they might exhibit different responses to pesticides due to differences in physiology and ecology (EFSA 2013).

Over the last decades, progress in research has been made in evaluating the risk that pesticides pose to bees by assessing the toxicological effects. Extensive empirical data have been accumulated and are showing that toxic effects vary depending on the dose/concentration, modes of action and exposure routes. However, a major improvement was to show that the effect of a given pesticide can be modulated by several endogenous and/or exogenous factors (Johnson, 2015; Poquet et al., 2016). Indeed, thanks to the diversity of methods used by researchers, the complex nature of pesticide toxicity is being revealed. At a given dose, pesticides do not induce one rigid effect but a range of responses that can vary in intensity depending on other factors (e.g. developmental stage, caste, genetic background). Such a modulation of response might not be surprising given that phenotypic

responses (e.g. development and behaviour) are generally regulated by both endogenous (genotype, physiology) and exogenous factors (environment). A comprehensive view of this response variability is therefore required to better understand the toxicity of pesticides and develop a more detailed pesticide risk assessment in bees.

For instance, several studies have shown that the genetic background in honey bees can influence their responses to pesticides, as evidenced by the differences in pesticide toxicity between honey bees originating from different subspecies (Ladas, 1972; Laurino et al., 2013; Rinkevich et al., 2015; Sandrock et al., 2014; Suchail et al., 2000). This may be explained by differences in the detoxification system. Indeed, when comparing different breeding stocks of honey bees, it was recently found that a lower pesticide tolerance was associated with a lower activity of esterase involved in xenobiotic detoxification (Milone et al., 2020). There are also clear physiological differences between castes of social bees (queen, drone, worker). However, few studies investigated their differential susceptibly to pesticides. By adjusting the body weight differences and comparing the LD_{50} values of honey bee queens and workers exposed to widely used acaricides, (Dahlgren et al., 2012) found that queens were more tolerant than workers to several acaricides. The underlying mechanisms are not known, but differences might arise from a caste-specific toxicokinetics of pesticides, since honey bee queens have different expression profiles of CYP450 family proteins and multidrug resistance proteins than workers (Chan et al., 2013). Both types of proteins are involved in pesticide resistance in insects (Buss et al., 2002; Li et al., 2007; Srinivas et al., 2004). Similarly, caste differences in the response to pesticides was found in the bumble bee *Bombus terrestris* (Mobley and Gegear, 2018). In addition, due to the widespread use of pesticides and their persistence in agrosystems, bees are often forced to cope with co-exposure to pesticides. Indeed, fungicides, herbicides and insecticides can all be found in the diet of bees. The toxicity of pesticides does not necessarily result in additive effects, but can be greater or lower than the sum of expected responses (Siviter et al., 2021).

Finally, there is a growing gap between new evidences of pesticide toxicity in bees (sublethal effects) and conventional toxicological assays recommended by regulatory test guidelines, which focus essentially on lethal endpoints. While sublethal effects do not directly cause the death of individual bees, they may impair the behavioural and reproductive performances of bees (honey bees, bumble bees, *Osmia*) (Meikle et al., 2016; Prado et al., 2019; Tison et al., 2017; Woodcock et al., 2017). This gap between new evidence for sublethal effects and the conservative toxicological bioassays contribute to the controversy between stakeholders, policymakers, environmentalists and scientists (Sgolastra et al., 2020; Storck et al., 2017; Thompson and Maus, 2007). In this context, we need to complement current endpoints (essentially based on LD_{50}) with sublethal endpoints, such as behavioural and reproductive performances, which are receiving growing interest due to their ecological relevance (Barascou et al., 2021).

Therefore, the goal of this deliverable was not only to provide data on the acute and chronic toxicity of pesticides, but also to give insights on the variability of responses based on endogenous (developmental stage, genetic background, caste) and exogenous factors (pesticide co-exposure). We also considered the risks posed by sublethal exposure to pesticides by implementing behavioural and reproductive endpoints in the screening of pesticide toxicity. These approaches were used to test the toxicity of sulfoxaflor, azoxystrobin and glyphosate (as well as cyantraniliprole and flupyradifurone) in three model bee species (*Apis mellifera*, *Bombus terrestris*, *Osmia bicornis*). They will therefore contribute to better understand the susceptibility of bees to pesticides.

We first used LD_{50} tests to determine the acute oral and contact toxicity of these pesticides across the different bee species, developmental stages (larva vs adult in honey bees), castes (honey bee and bumble bee workers, queens and drones), and genetic backgrounds (honey bee subspecies) (PoshBee Task 3.1). Then, mortality, behavioural and reproductive endpoints were used to assess the chronic

toxicity of different pesticides (PoshBee Task 3.3). Finally, to give insights on the outcome of pesticide co-exposure, the potential interaction effects among pesticide classes were investigated (PoshBee Task 3.4).

2. Assessment of oral and contact LD_{50}

2.1. Honey bee (*Apis mellifera*)

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The contact and oral dose-responses for sulfoxaflor, azoxystrobin and glyphosate were determined in honey bee (*Apis mellifera* L.) workers and larvae. The methods used in these experiments have been described and published under PoshBee Deliverable D3.2.

Experiments were performed in four *A. mellifera* subspecies in four different countries: subspecies *iberiensis* in Spain, subspecies *ligustica* in Italy, subspecies *carnica* in Sweeden and subspecies *mellifera* in Switzerland. Each country selected three honey bee colonies as replicates for the experiments.

2.1.1. Development stages

2.1.1.1. Adults

Oral dose-response relationship
The improved protocols for testing agrochemicals in honey bees were used (see D3.2). Briefly, flying honey bees (foragers) were collected from each colony and subsequently anaesthetized by CO_2:air mixture (3:2 to 2:1 v/v). Then, honey bees from the same colony were randomly allocated to test cages. Each cage contained 20 honey bees that were fed with 200 microliters of the test feeding solution. Pure sucrose solution was provided *ad libitum* once the test feeding solution was finished. The test temperature was 25°C ± 1°C and mortality was assessed at 4, 24 and 48 hours post-exposure.

The LD_{50} was calculated for sulfoxaflor as active pure substance. The assessment of LD_{50} was performed with seven increasing doses (from 0.009375 to 0.6µg/bee). Two negative controls (one with water and one with acetone) and one reference chemical (dimethoate) were included in the experiment (see D3.2 for more details). Results obtained by each subspecies are shown in Figures 1 to 4.

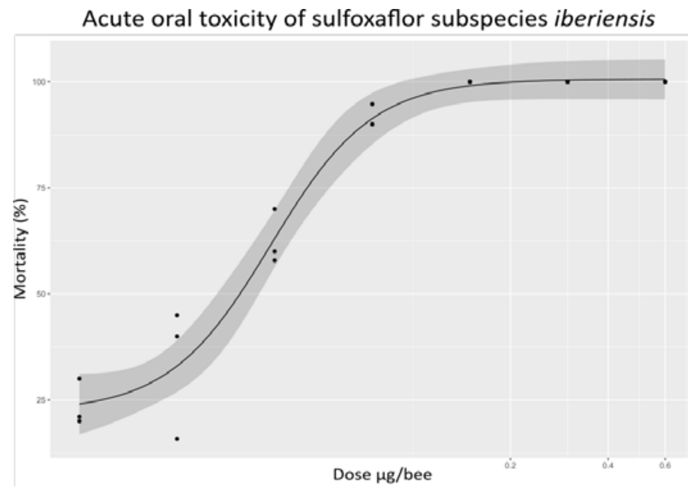

Figure 1. Acute oral toxicity of sulfoxaflor (48h) in the subspecies *iberiensis*

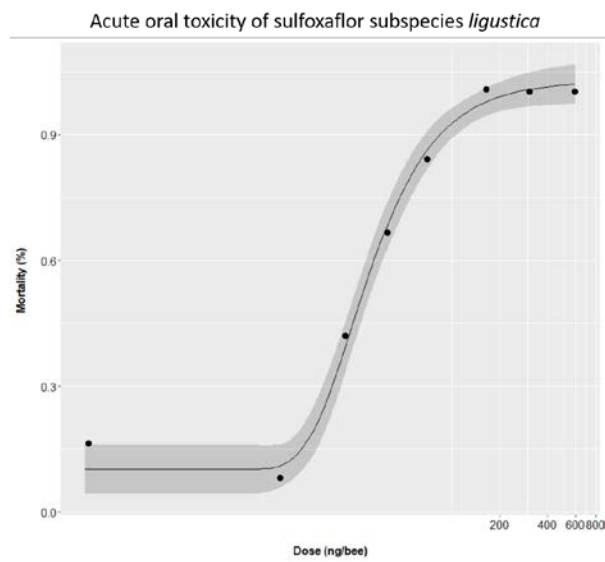

Figure 2. Acute oral toxicity of sulfoxaflor (48h) in the subspecies *ligustica*

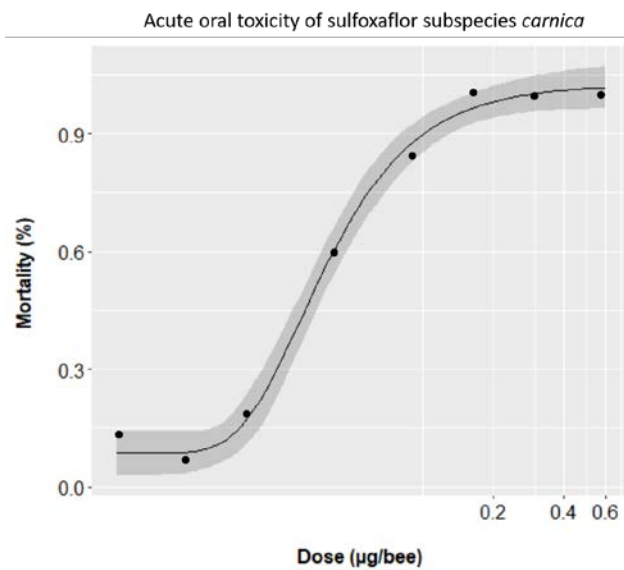

Figure 3. Acute oral toxicity of sulfoxaflor (48h) in the subspecies *carnica*

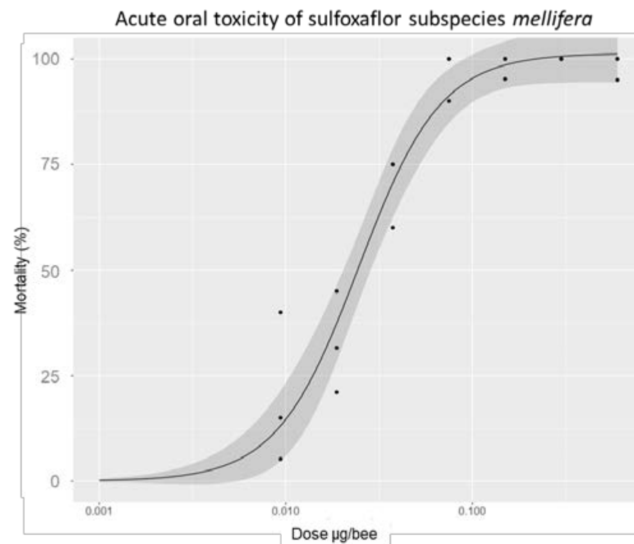

Figure 4. Acute oral toxicity of sulfoxaflor (48h) in the subspecies *mellifera*

The LD_{50} was calculated for azoxystrobin as the commercial formulation Amistar The assessment of LD_{50} was performed with five increasing doses (from 6.25 to 100µg/bee). One negative control with water and one reference chemical (Dimethoate) were included in the experiment (see D3.2 for more details). Results obtained for each subspecies are shown in Figures 5 to 7.

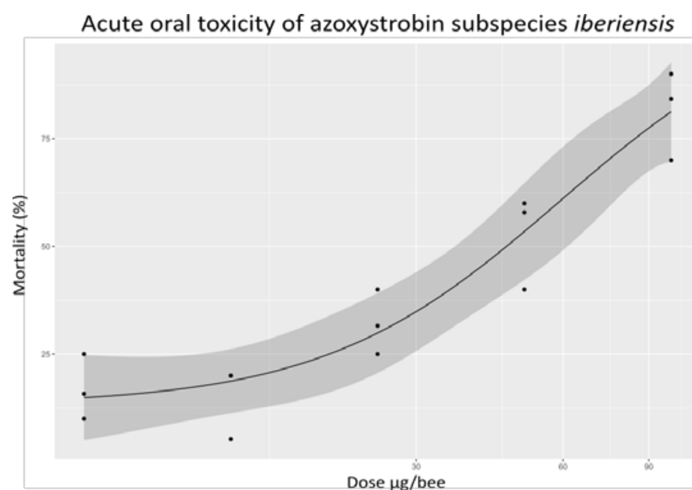

Figure 5. Acute oral toxicity of azoxystrobin (48h) in the subspecies *iberiensis*

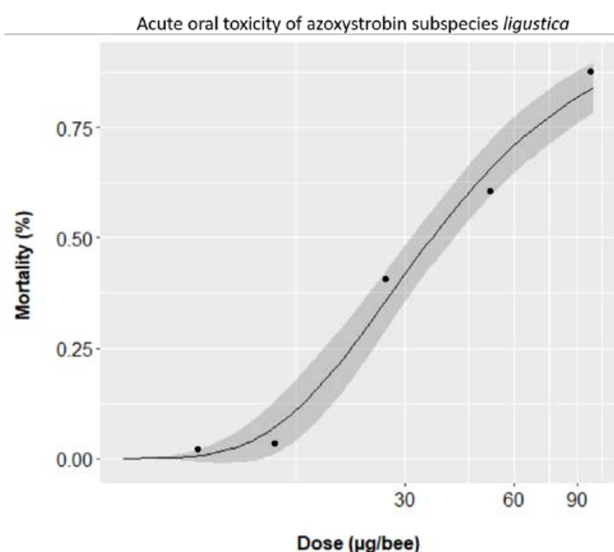

Figure 6. Acute oral toxicity of azoxystrobin (48h) in the subspecies *ligustica*

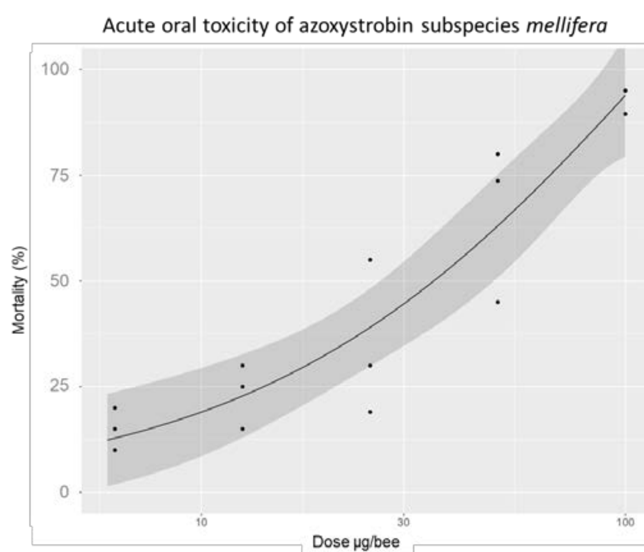

Figure 7. Acute oral toxicity of azoxystrobin (48h) in the subspecies *mellifera*

Due to the low mortality levels observed for the *carnica* subspecies during the LD_{50} test, a limit test was carried out using azoxystrobin (100 µg/bee), one control solution with water and one reference chemical (dimethoate). The azoxystrobin dose caused 25.67±2.69 % mortality.

Toxicity of glyphosate was tested with a commercial formulation Roundup Platinum. Limit tests instead of LD_{50} were used given the low solubility of the substance in water and the low presumed toxicity. Thus, the experiment included three solutions: glyphosate (100 µg/bee), one control solution with water and one reference chemical (dimethoate) (see https://poshbee.eu/documents/1/D3.2 for more details). Results obtained by each subspecies are shown in Table 1.

Table 1: Acute oral toxicity of gyphosate (100 µg/bee) in the four tested subspecies: *carnica*, *iberiensis, ligustica* and *mellifera* at 48h post-exposure.

	carnica	*iberiensis*	*ligustica*	*mellifera*
Mortality (%)	5.09±3.33	20.17±4.59	18.03±4.70	18.33±5.04

Contact dose-response relationship

The improved protocols for testing agrochemicals in bees were used (see D3.2). Flying honey bees (foragers) were collected from each colony and subsequently anaesthetized by CO_2:air mixture (3:2 to 2:1 v/v). Then, honey bees from the same colony were randomly allocated to test cages. Each cage contained 20 honey bees that were treated individually with 1µl of treatment solution. Pure sucrose solution was provided *ad libitum*. The test temperature was 25°C ± 1°C and mortality was assessed at 4, 24 and 48 hours post-exposure.

The LD_{50} was calculated for sulfoxaflor as the pure active substance. The assessment of LD_{50} was performed with five increasing doses (from 0.009375 to 1.5µg/bee). Mortality in the subspecies *mellifera* was too high and therefore the test was repeated with lower dosages (from 0.0117 to 0.1875 µg/bee). Two negative controls (one with water and one with acetone) and one reference chemical (dimethoate) were included in the experiment (see D3.2 for more details). Results obtained by each subspecies are shown in Figures 8 to 11.

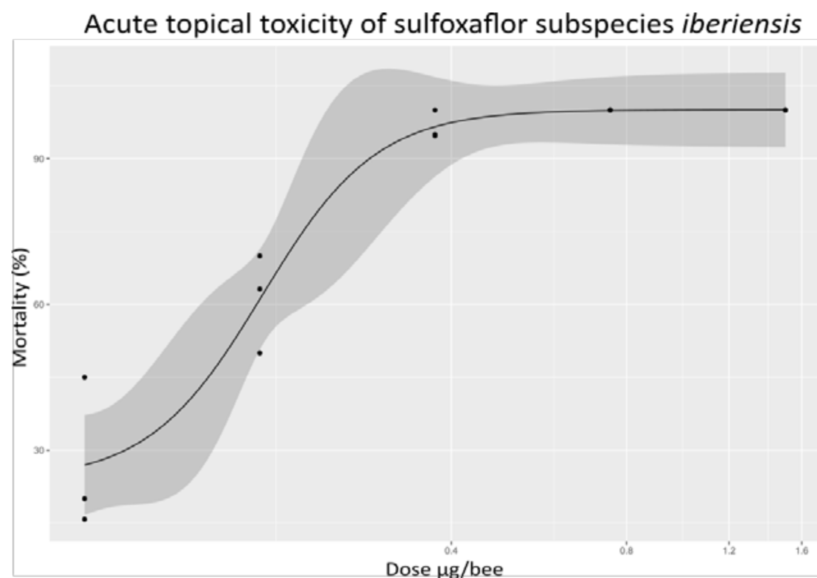

Figure 8. Acute topical toxicity of sulfoxaflor (48h) in the subspecies *iberiensis*

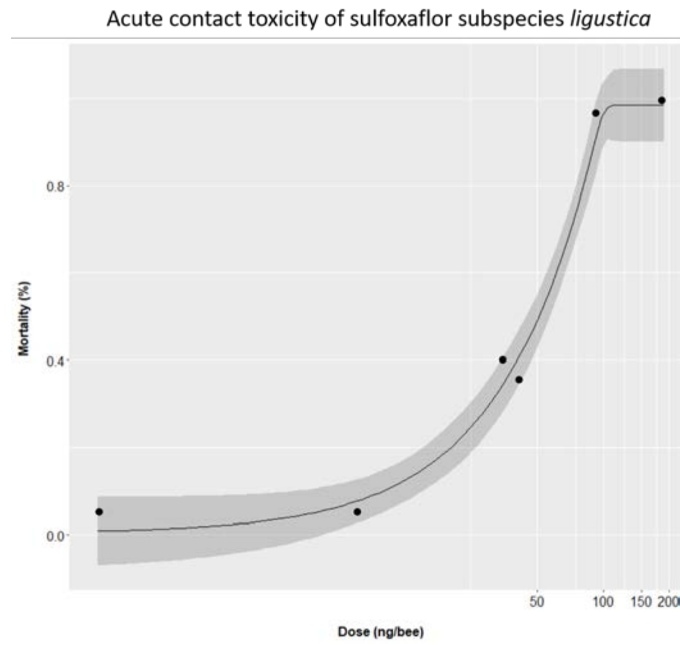

Figure 9. Acute topical toxicity of sulfoxaflor (48h) in the subspecies *ligustica*

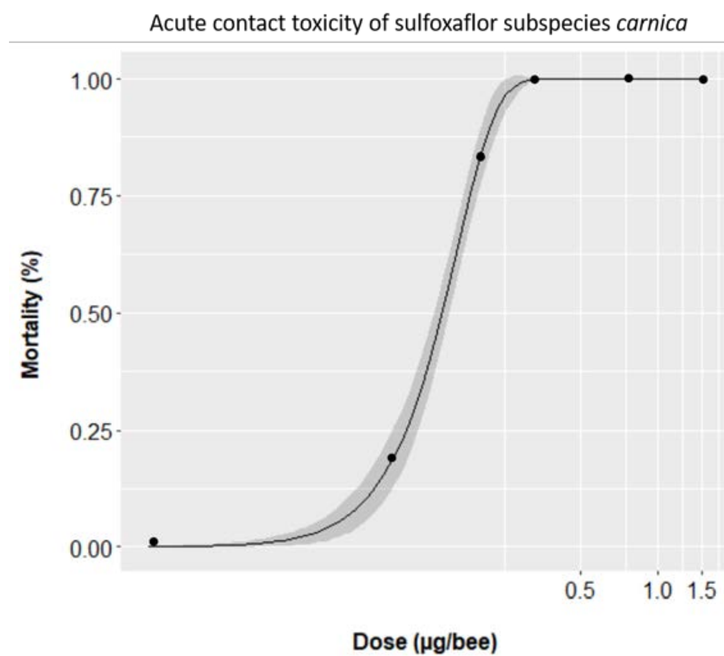

Figure 10. Acute topical toxicity of sulfoxaflor (48h) in the subspecies *carnica*

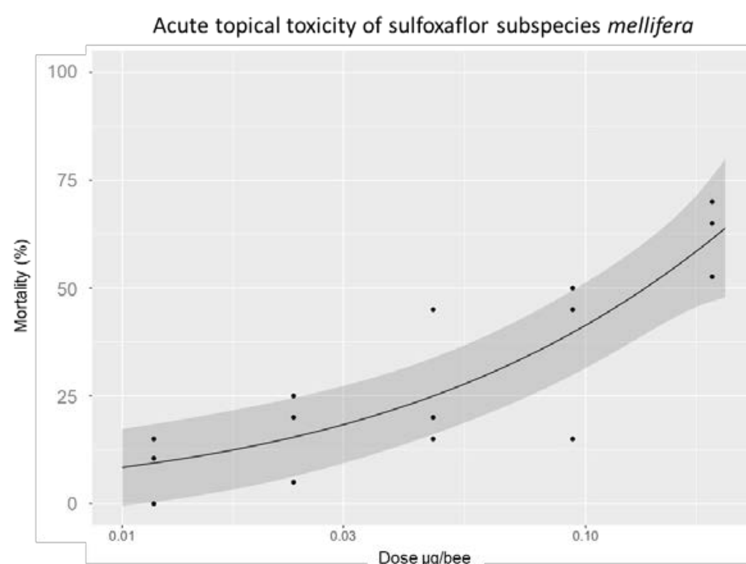

Figure 11. Acute topical toxicity of Sulfoxaflor (48h) in the subspecies *mellifera*

The toxicity of azoxystrobin and glyphosate was tested with the commercial formulations Amistar and Roundup Platinum, respectively. Limit tests instead of LD_{50} were used given the low solubility of these substances in acetone and the low presumed toxicity. Thus, the experiment included three solutions: azoxystrobin or glyphosate (100 µg/bee), one control solution with water and one reference chemical (dimethoate) (see D3.2 for more details). Results obtained by each subspecies are shown in Table 2.

Table 2 Acute topical toxicity of azoxystrobin and glyphosate (100 µg/bee for both pesticides) in the four tested subspecies: *carnica*, *iberiensis, ligustica* and *mellifera* at 48h post-exposure.

Mortality (%)	*carnica*	*iberiensis*	*ligustica*	*mellifera*
Azoxystrobin	1.75±2.02	18.59±2.62	1.7±1.67	13.33±4.43
Glyphosate	1.67±1.92	15.26±2.37	6.7±2.78	3.33±2.34

2.1.1.2. Larvae

The methodology is based on the OECD TG 237 (OECD 2013) in conjunction with the adapted protocols described in D3.2. Briefly, between 12 and 16 larvae of the same age (three days) per testing solution were collected for each one of the three replicates. The test temperature was kept between 34 and 35°C and mortality was assessed at 24, 48 and 72 hours post-exposure. For each test, two negative controls (one with pure diet C as described in OECD 2013 and one with acetone) and one reference chemical (dimethoate) were included in the experiment (see D3.2 for more details).

The LD_{50} was calculated for sulfoxaflor as the pure active substance. The assessment of LD_{50} was performed with five increasing doses (from 0.0015 to 15 µg/larva for *iberiensis*, and from 2.81 to 45 µg/larva for *carnica*, *ligustica* and *mellifera*). Results obtained by each subspecies are shown in Figures 12 to 15.

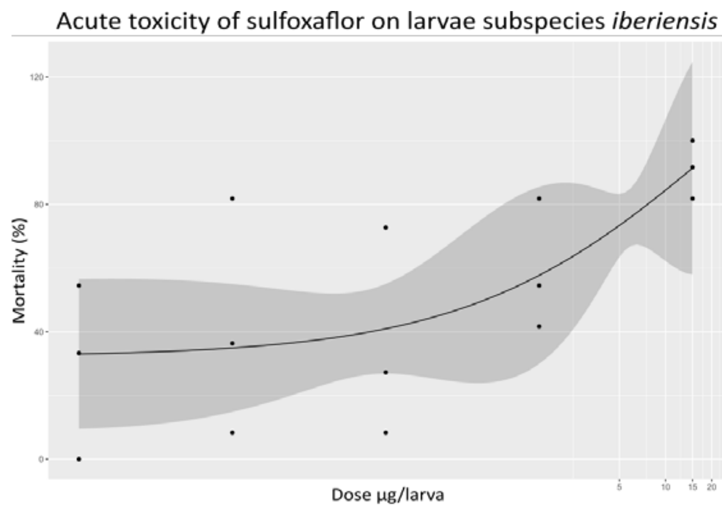

Figure 12. Acute toxicity of sulfoxaflor on larvae (72h) in the subspecies *iberiensis*

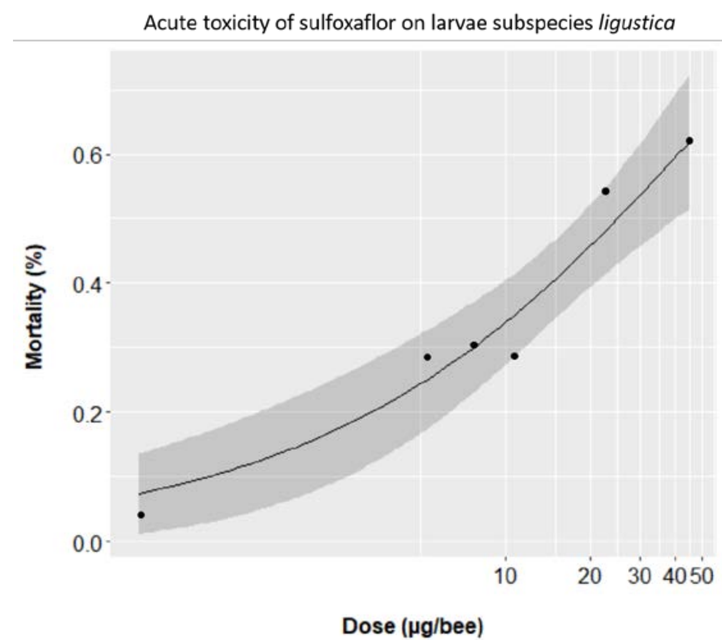

Figure 13. Acute toxicity of sulfoxaflor on larvae (72h) in the subspecies *ligustica*

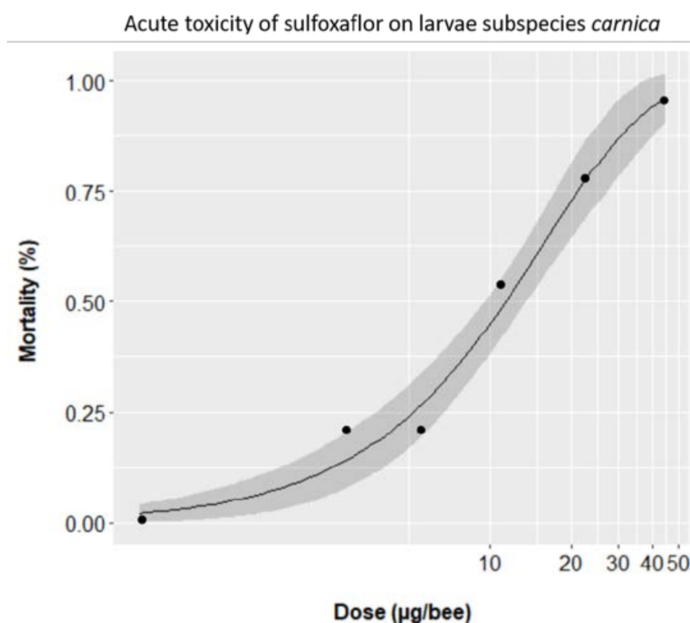

Figure 14. Acute contact toxicity of sulfoxaflor on larvae (72h) in the subspecies *carnica*

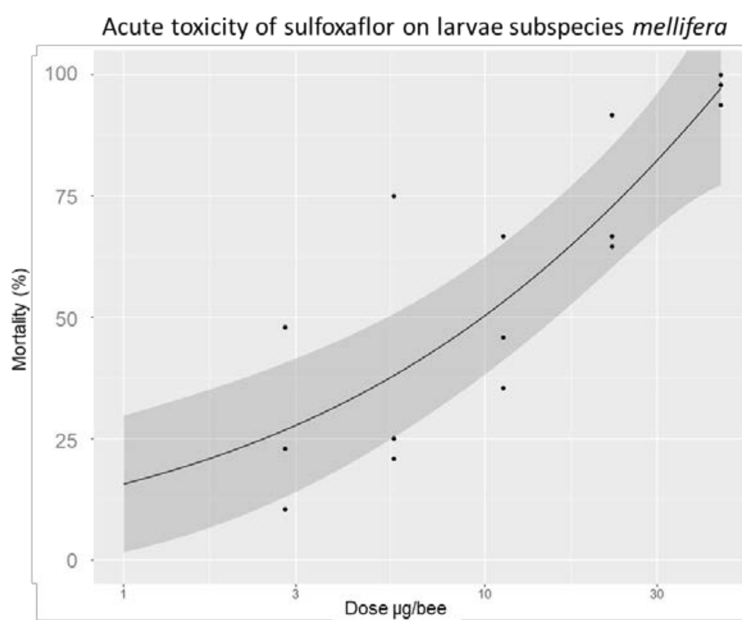

Figure 15. Acute toxicity of sulfoxaflor on larvae (72h) in the subspecies *mellifera*

The LD_{50} was calculated for azoxystrobin as the pure active substance. The assessment of LD_{50} was performed with five increasing doses (from 0.0384 to 24 µg/larva). As is shown in Figure 16, LD_{50} could not be calculated for *iberiensis* due to the low toxicity of the substance.
Due to the low mortality levels observed during the Azoxystrobin LD_{50} test, limit tests were carried out using 72 µg/larva for the subspecies *carnica* and *ligustica*, and 24 µg/larva for the subspecies *mellifera* (Table 3).

Table 3. Acute oral toxicity of azoxystrobin (72 µg/larva *carnica* and *ligustica*, and 24 µg/larva *mellifera*) and glyphosate (30 µg/larva for *carnica, ligustica and mellifera*) at 72h post-exposure.

Mortality (%)	*carnica*	*ligustica*	*mellifera*
Azoxystrobin	19.34±1.95	5.56±1.91	34.72±3.98
Glyphosate	0	2.08±2.08	1.38±0.98

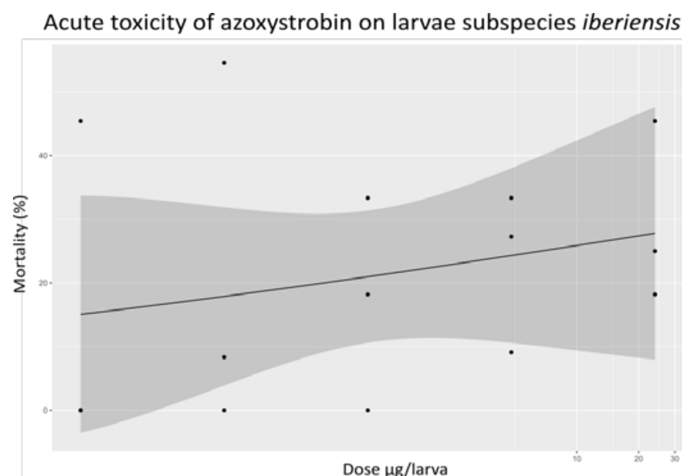

Figure 16. Acute toxicity of azoxystrobin on larvae (72h) in the subspecies *iberiensis*

The LD_{50} was calculated for glyphosate as the pure active substance. The assessment of LD_{50} was performed with five increasing doses (from 0.048 to 30 µg/larva). As is shown in Figure 17, LD_{50} could not be calculated for *iberiensis* due to the low toxicity of the substance. Furthermore, mortality in the subspecies *iberiensis* was poorly correlated with the dose of glyphosate tested which may be due to a background exposure to the substance in the area where there are many crops and gardens.

Due to the low mortality levels observed for the *carnica*, *ligustica* and *mellifera* subspecies during the glyphosate LD_{50} tests, limit tests were carried out using 30 µg/larva (Table 3).

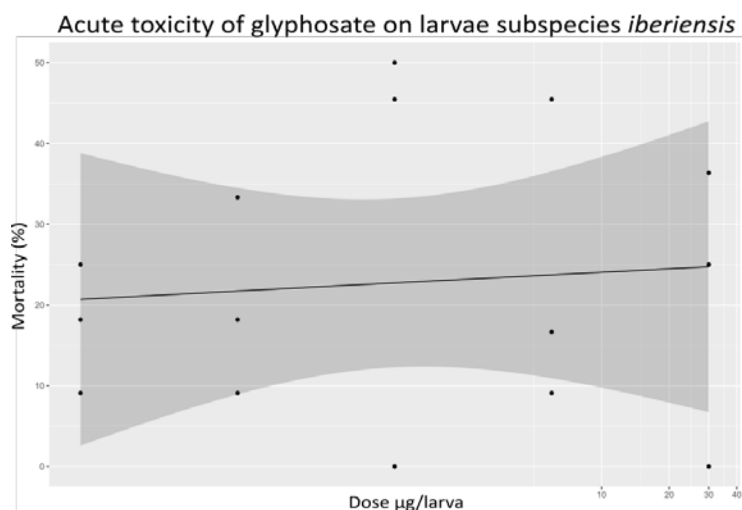

Figure 17. Acute toxicity of Glyphosate on larvae (72h) in the subspecies *iberiensis*

2.1.2. Reproductive castes

2.1.2.1. Queens

Experiments on queens will be performed in June - July 2022 by BERN.

2.1.2.2. Drones

Oral dose-response relationship

Only pre-trials were performed to find the best method for exposing drones without workers. Actual experiments will be performed in May 2022 by BERN.

Contact dose-response relationship

Newly emerged drones were used for this experiment. Following the same protocol as for workers (see above), drones were topically exposed to the different chemicals. After exposure drones were kept in cages (10 drones each) together with 20 workers to maintain drone attendance. The LD_{50} was calculated for sulfoxaflor as the pure active substance. The assessment of LD_{50} was performed with five increasing doses (from 0.0117 to 0.1875 µg/bee). Two negative controls (one with water and one with acetone) and one reference chemical (dimethoate) were included in the experiment. The result is shown in Figure 18.

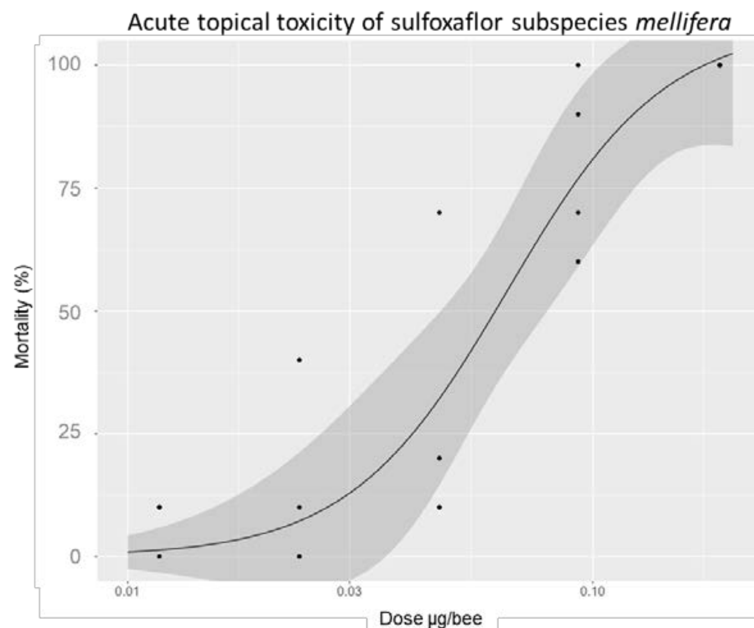

Figure 18. Acute topical toxicity of sulfoxaflor (48h) in drones of the subspecies *mellifera*

The toxicity of azoxystrobin and glyphosate was tested with commercial formulations Amistar and Roundup Platinum, respectively. Limit tests instead of LD_{50} were used given the low solubility of these substances in acetone and the low presumed toxicity. Thus, the experiment included four solutions: azoxystrobin and glyphosate (100 µg/bee), one control solution with water and one reference chemical (dimethoate). Azoxystrobin and glyphosate causes 2.5±42.53 and 5±3.49 % mortality, respectively at 48 hrs post-exposure.

2.1.3. Genetic backgrounds

Samples of worker honey bees and drones were received from Italy (CREA), Switzerland (BERN), Sweden (SLU) and Spain (UM). Individuals were preserved in ethanol or in RNA-later (the latter for transport and customs reasons) and kept at -20°C until processing. Genomic DNA was extracted from

individual legs of a single honey bee worker per colony. Evolutionary lineages and haplotypes were identified by a method based on the variation in the mitochondrial intergenic region located between the tRNA-leu and *cox*2 genes (Garnery et al., 1993). Genotypes generated with informative SNPs (Henriques et al., 2018a; Henriques et al., 2018b) were used for admixture ancestry through population structure assessment and introgression proportions analyses.

Three evolutionary lineages were detected: African (A-lineage), West Mediterranean and North European (M-lineage) and Central and Southeast European (C-lineage). In Spain, only haplotypes from the A-lineage (A1 and A2) were detected (Fig. 19). In Italy all samples belonged to the C-lineage and showed the same haplotype (C1a) whereas in Switzerland, only one haplotype (M4) from the M-lineage was detected. Finally, In Sweden 42.9% of the samples belonged to the M-lineage bearing haplotypes M4 and M4' while 57.1% belonged to the C-lineage (C2e).

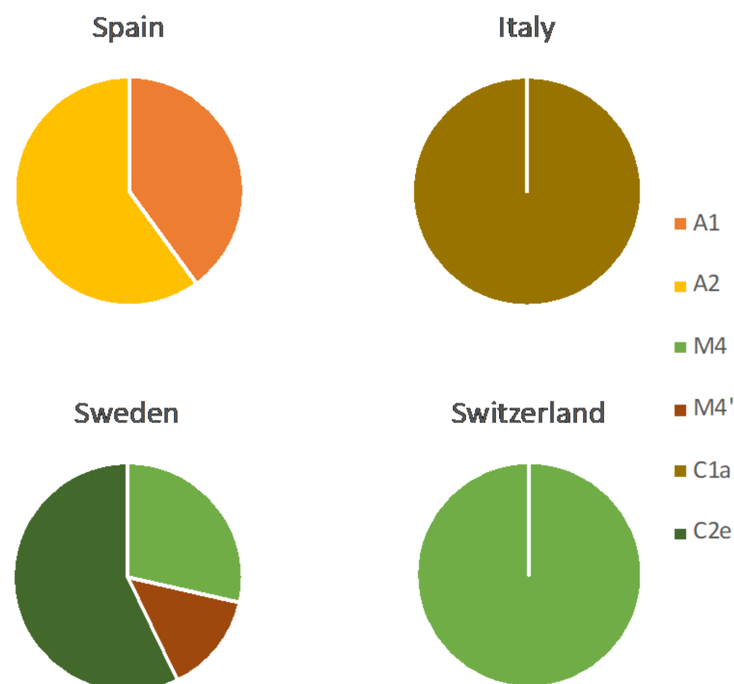

Figure 19. Frequency of haplotype detected in the four countries included in the study.

Membership proportions (Q) were inferred from the SNP-genotypes using the software STRUCTURE (Fig. 20). The ΔK method (Evanno et al., 2005) indicated that K = 3 was the most likely number of genetic clusters for the *mellifera* panel, however K = 2 was the most likely number of clusters for the *iberiensis* panel. The *mellifera* panel revealed three main clusters: cluster-1 in the Iberian Peninsula corresponding to the subspecies *iberiensis*, cluster-2 corresponding to the subspecies *mellifera*, and cluster-3 formed by the samples from Italy where the subspecies *ligustica* occurs and by those samples from Sweden with the C2e haplotype (characteristic of the subspecies *carnica*), making cluster-3 representative of the C lineage.

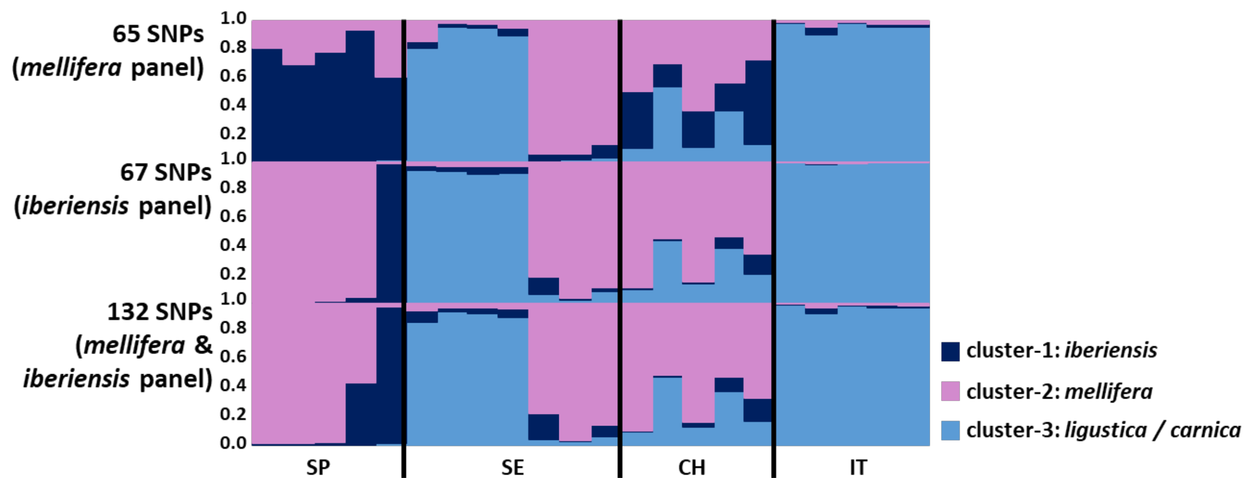

Figure 20. STRUCTURE plot for K = 3 for the entire data set. A mean assignment probability range of 0.1 < q < 0.3 indicates a weak admixture, while a range of 0.7 < q < 0.9 means high admixture.

If we consider each cluster derived by the SNP-genotypes as the local subspecies of each country (Fig. 21), then the colonies from Italy correspond to *A. m. ligustica* and show a haplotype characteristic of this subspecies and the lowest admixture level. Colonies from Spain correspond to *A. m. iberiensis* and show a level of admixture ranging from 0.6 to 0.9 from *mellifera*, an expected result given the hybrid origin of this subspecies between European *mellifera* and African *intermissa* subspecies. Swedish colonies resemble two subspecies, the local *A. m. mellifera* and the introduced *A. m. carnica* with almost no admixture between the two subspecies. On the contrary, the colonies from Switzerland, although bearing one unique haplotype, show a complex admixture pattern with samples showing SNPs more frequently observed in *mellifera*, two samples with a high admixture level from *iberiensis* and the other two from *ligustica*.

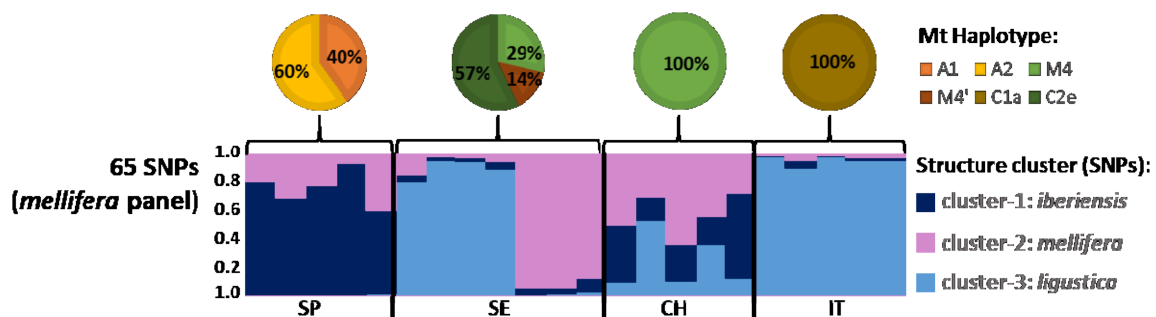

Figure 21. Haplotype distribution and frequency in each of the three clusters (resembling subspecies) inferred from the SNPs *mellifera* panel.

2.2. Bumble bee (Bombus terrestris)

Margret Jurison, Marika Mand (EMU), Alberto Linguadoca, Harry Siviter, Morgan Morrison, Antoine Gekière, Ferozah Mahmood, Edward A Straw and Mark JF Brown (RHUL)

2.2.1. Oral dose-response relationships

The mortality hazards of sulfoxaflor, Amistar (azoxystrobin 250g/l SC) and glyphosate to the bumble bee (*Bombus terrestris*) were tested using a dose-response design. We re-adapted a standard methodology (OECD 2017) to test pesticide toxicity in workers, males and queens (see deliverable

D3.2). Bees were sourced from a local supplier (Agralan, UK). Workers were taken from queenright colonies, while males were either taken from queenright colonies (Agralan, UK, 1 experiment) or queenless bee boxes (Agralan, UK, all other experiments). Additionally, we sourced colonies at the onset of sexual brood production (Koppert, SK) to take unmated queens.

We used a feeding method where bees were individually housed in Nicot cages (Nicotplast, FR) and fed an acute (40 µL) provision of pesticide-spiked sucrose solution (30% w/w). Whenever possible (*i.e.*, in all experiments, except those using queenless male bee boxes) bees were allocated to the treatment by colony of origin and size. Particularly small or large bees were excluded (OECD 2017). Twenty-four hours after housing, bees were exposed to either the untreated or treated syrup.

Treatments were prepared by means of serial dilutions of a water-based stock solution of either sulfoxaflor (ChemService, USA) or glyphosate (Pestanal, Sigma Aldrich, UK). For Amistar, treatments were prepared by means of serial dilutions of the undiluted formulation, which was used as a stock solution (Amistar, Syngenta, UK, azoxystrobin 250 g/L SC).

Each experiment consisted of an untreated control, a positive control consisting of a 4 µg/bee dose of dimethoate (Pestanal, Sigma Aldrich, UK) and a minimum of 5 increasing doses of sulfoxaflor (Table 4) spaced by a geometric factor not exceeding 2. Dose selection was informed by range finding tests (results not shown). Across experiments, no organic solvent, wetting agent or emulsifier were used. Worker and male bumble bees were fed a 40 µL droplet of treated or untreated sucrose solution. Two to four hours after administration, consumption was visually checked and bees who did not consume the entire provision of syrup were excluded from the test. Upon exposure, mortality was recorded at 6h, 24h and 48h. If evidence of delayed toxicity was observed, the observations were prolonged to 96h.

Table 4. Treatments in the bumble bee acute oral toxicity tests.

	Test item	**N (initial)**	**N (after exclusion of non-feeders)**	**Mean mass mg ±SE**	**Treatment dose per bee (µg a.s./bee)**
Workers	Sulfoxaflor	35	29-35	211 (10)	0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18
Males	Sulfoxaflor	30-31	22-25	232 (11)	0.02, 0.06, 0.1, 0.14, 0.18, 0.22
Queens	Sulfoxaflor	40	17-26	619 (29)	0.09, 0.18, 0.36, 0.72, 1.44
Workers	Amistar	30	20-29	239 (6)	40, 80, 160, 320, 640, 1280
Males	Amistar	37	15-33	285 (9)	40, 80, 160, 320, 640, 1280
Queens	Amistar	45	7[1]-36	731 (29)	175, 350, 700, 1400, 2800
Workers	Glyphosate	Results were produced by RHUL as part of WP6, showing no mortality at a limit dose of 200 µg/bee in the same setup described in this section.			
Males	Glyphosate	60	33	295 (9)	200
Queens	Glyphosate	55	18	727 (24)	200

[1]*the highest tested dose was clearly avoided by queens*

Dimethoate always caused mortality rates higher than 50%, indicating that our system was adequately sensitive. No mortality was observed across experiments in the untreated group.

Sulfoxaflor

Males were the most sensitive to sulfoxaflor, followed by workers and queens (Figure 22). The LD_{50s} across bumble bee castes and sexes are reported in Table 5.

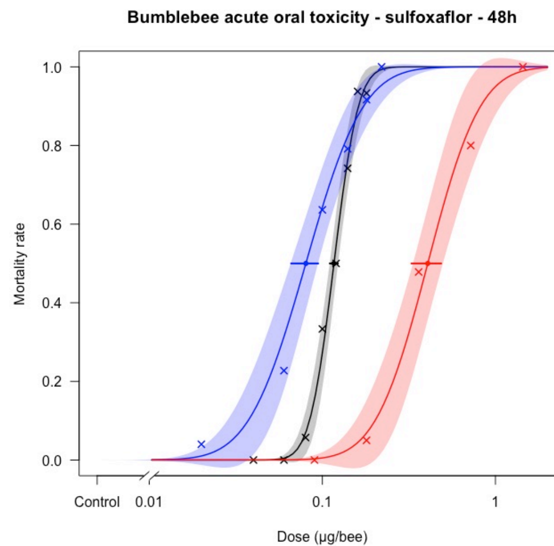

Figure 22. Acute oral toxicity of sulfoxaflor in bumble bee males (blue), workers (black) and queens (red). The 2-parameter log normal dose-response is described by a sigmoidal curve, along with the 95% confidence band (shaded area). The x marks indicate observed mortality level at each tested concentration 48 hrs post-exposure. The LD_{50} and 95% CI are described by points and horizontal segments respectively.

Amistar

Given that we found evidence of delayed mortality upon exposure to Amistar, the test was prolonged to 96h. As for sulfoxaflor, males were found to be the most sensitive, followed by workers and queens (Figure 23). The LD_{50s} across bumble bee castes and sexes are reported in Table 5.

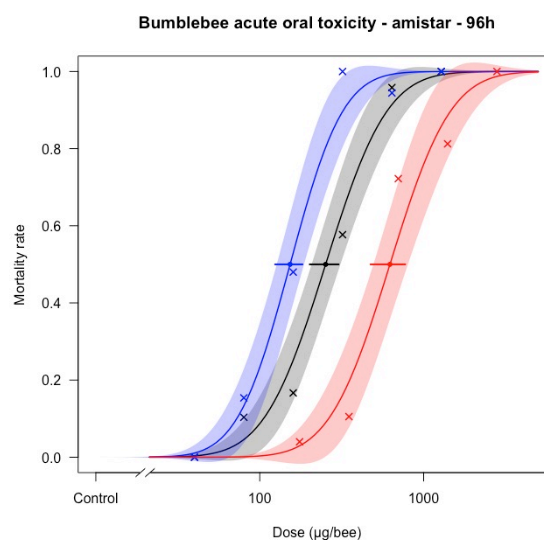

Figure 23. Acute oral toxicity of amistar in bumble bee males (blue), workers (black) and queens (red). The 2-parameter log normal dose-response is described by a sigmoidal curve, along with the 95% confidence band (shaded area). The x marks indicate observed mortality levels at each tested concentration 96 hrs post-exposure. The LD_{50} and 95% CI are described by points and horizontal

segments respectively. Bees were exposed to the formulated product. However, the reported dose refers to the quantity of active ingredient.

Table 5. Acute oral LD_{50} (95% CI) of sulfoxaflor and Amistar across bumble bee castes and sexes calculated via probit regression.

Assessment time	Test item	Workers	Males	Queens
		µg/ bee	µg/ bee	µg/ bee
48 h	sulfoxaflor	0.117 (0.11 – 0.123)	0.080 (0.064 – 0.095)	0.406 (0.331 – 0.503)
96 h	amistar	252 (206 – 312)	153 (94 – 269)	623 (489 – 816)

Glyphosate

Glyphosate did not cause significant mortality in workers, males, or queens (Fisher's exact test, p=1, mortality rate = 3% and 0% respectively). Therefore, the NOEL was set at 200 µg/bee and the LD_{50} was confirmed to be higher than the tested dose.

2.2.2. Contact dose-response relationships

The mortality dose-responses upon contact exposure to sulfoxaflor, azoxystrobin and glyphosate were evaluated for bumble bee (*Bombus terrestris* L.) workers, males and queens. The test methodologies were developed based on the OECD guidelines (OECD 2017) for acute contact toxicity. The methods used in these experiments have been described and published under Deliverable D3.2.

B. terrestris was purchased from a local supplier A.M. OZOLI (Cīruļdārzi, Eimuri, LV-2164, Latvia). Bumble bee workers were purchased as boxed queen-right colonies, males were purchased as queenless boxes, and queens were bought as individual specimens. Bumble bees were taken from five different colonies or packages. All the bumble bees were randomly selected and evenly distributed by weight (avoiding extremely small and large sized workers). After selection, bees were individually allocated to Nicot® cages and acclimatised to the test conditions (25 ± 1 °C, ~60% relative humidity and darkness) for 24 h with access to *ad libitum* and untreated 50 % weight per volume (w/v) aqueous sucrose solution.

Each experiment consisted of (1) undosed control, (2) positive control (dimethoate) and (3) different dilutions of pesticide or one limit test dose (Table 6). For stock solutions 10 mg of sulfoxaflor (ChemService, purity 99.4% in powder form) and 100 mg of azoxystrobin (Honeywell Fluka, purity 98% in powder form) were dissolved in 0.2 ml and 2 ml of acetone, respectively. The low water solubility (about 10g/L) of glyphosate made the determination of the LD_{50} impossible. Instead, limit tests were carried out with the commercial formulation product (RoundUp FL) that contains 450g of active ingredient per L of the formulation.

Worker and male bumble bees were exposed to a 2 µL droplet of the tested pesticide, and queens to a 4 µL droplet. Before administering the droplets, 0.1% of triton-X-100 was added to the final solutions. Bees were viewed as dead when they did not move their legs or antennae and did not respond to provocation. Mortality data were recorded at 6, 24, 48 hours after treatments. If evidence of delayed toxicity was observed, the observations were prolonged to 96h.

Table 6. Treatments in the bumble bee acute contact toxicity tests.

	Test item	N	Mean mass mg	Treatment dose per bee (µg/bee)
Workers	Sulfoxaflor	46	237	50, 25, 10, 5, 1, 0.1
Males	sulfoxaflor	40	330	25, 16, 10, 5, 1, 0.1
Queens	Sulfoxaflor	30	782	400, 200, 100, 50, 20, 5
Workers	Azoxystrobin	50	271	100
Males	Azoxystrobin	50	309	100
Queens	Azoxystrobin	30	915	100
Workers	Roundup FL	45	239	200
Males	Roundup FL	49	278	200
Queens	Roundup FL	20	873	200

Dimethoate always caused mortality rates higher than 50%, indicating that our system was adequately sensitive. No mortality was observed across experiments in the untreated group.

As presented in Figure 24, male bumble bees were the most susceptible to sulfoxaflor (LD_{50} 48 h: 0.679 µg/bumble bee, 95% confidence limits: 0.443 - 0.916). They were followed by workers (LD_{50} 48 h: 6.323 µg/ bumble bee, 95% confidence limits: 4.672 - 7.974). The queens were the most resistant to Sulfoxaflor (LD_{50} 48 h: 75.241 µg per bumble bee, 95% confidence limits: 56.808 - 93.675). Tested azoxystrobin and glyphosate, did not cause significant mortality in workers, males and queens after 48 h of exposure. The LD_{50} values for 48h are shown in Table 7.

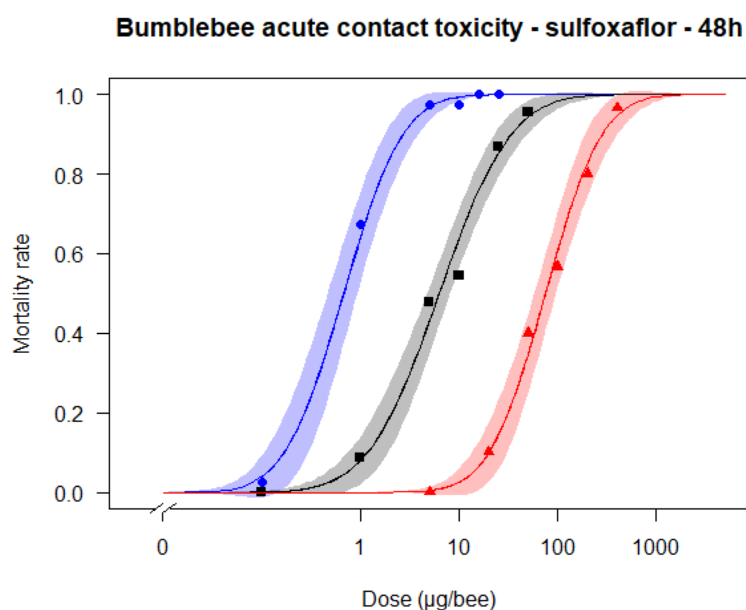

Figure 24. Acute contact toxicity of sulfoxaflor in bumble bee males (blue), workers (black) and queens (red). Bumble bee mortality curves are indicated by a solid line, 95% confidence limits by filled area. The dots indicate observed mortality for each tested dose 48 hours post-exposure.

Table 7. LD_{50} values for *B. terrestris* workers, males and queens contact exposure in µg/bee and ng/mg bodyweight (95% CI) after 48 h of exposure.

Test item	Caste	n/treatment	µg/bee (C.I.)	ng/mg (C.I.)
Sulfoxaflor	Workers	46	6.323 (4.672 - 7.974)	26.679 (19.712 - 33.646)
	Males	40	0.679 (0.443 - 0.916)	2.058 (1.342 - 2.776)
	Queens	30	75.241 (56.808 - 93.675)	96.216 (72.645 - 119.789)
Azoxystrobin	Workers	50	>100 µg	NA
	Males	50	>100 µg	NA
	Queens	30	>100 µg	NA
Roundup FL	Workers	45	>200 µg	NA
	Males	49	>200 µg	NA
	Queens	20	>200 µg	NA

2.3. Solitary bee (*Osmia bicornis*)

Sara Hellström, Robert Paxton (MLU)

The methodologies for handling, exposing and housing *Osmia bicornis* males and females are described in D3.2. Experiments were conducted at Martin Luther University in Halle, Germany. Full LD_{50} defining experiments were performed for sulfoxaflor contact and oral, and for azoxystrobin oral in its formulation Amistar. For glyphosate contact and oral and azoxystrobin contact, only limit tests were performed. One replicate of each experiment was analysed. All experiments were performed on males and females separately using identical methods and dosing regimes. *Osmia bicornis* males and females in diapause were shipped from the commercial rearer Pollinature GhmB (Konstanz, Germany).

All doses used in experiments are presented in Table 8. Analytical-grade sulfoxaflor was dissolved in acetone and subsequently serially diluted in acetone to the desired test concentration. For oral tests, the desired concentration was added to a 25% v/w sugar solution, creating a test solution with 1% acetone solvent. For topical exposure, acetone solvent containing the compound was used directly on the thorax of the animal. For azoxystrobin, the commercial formulation Amistar (azoxystrobin 250 g/l, UK Syngenta, 250 g/L a.i) was diluted in distilled water to the desired concentration and mixed 1:1 with 50% w/v sugar solution in order to achieve an oral test solution containing 25% w/v sugar. The commercial formulation was used due to the pure compound being insoluble in a sugar/water matrix. For contact azoxystrobin, pure compound in powder form was solved in acetone and diluted to the desired concentration. For glyphosate oral exposure, the commercial formulation Roundup ProActive (Monsanto, 480 g/L a.i.) was diluted in distilled water to the desired concentration and mixed 1:1 with 50% w/v sugar solution in order to achieve an oral test solution containing 25% w/v sugar. For contact glyphosate exposure, the same product dissolved in water with surfactant 0.1% Triton X added was used. Dimethoate dissolved in acetone was used as a positive control in all trials, along with a negative control and 1% acetone solvent control when relevant.

Table 8. Doses of active ingredients used in acute experiments with *Osmia bicornis*

Compound	Route	Doses (µg/bee)
Sulfoxaflor	Oral	0, 0.039, 0.02, 0.01, 0.005, 0.0025
Sulfoxaflor	Contact	0, 0.39, 0.2, 0.1, 0.05, 0.025
Azoxystrobin	Oral	0, 724.48, 362.24, 181.12, 90.56, 45.28
Azoxystrobin	Contact	000
Glyphosate	Oral	100
Glyphosate	Contact	100

Since no standard validity criteria for *O. bicornis* regulatory tests exists, it was decided to count tests with <15% control mortality at the desired time point as valid (Medrzycki et al., 2013). Using this criterion, tests for sulfoxaflor female oral exposure at 96h and 72h were not valid and were therefore excluded from further analysis. Differences in mortality rates between negative and solvent control were tested using Fisher's Exact test, and combined in further analyses as the tests were not significant ($p > 0.05$). Mortality correction formula for unequal samples was applied in cases where control mortality was between 5-15%. All datasets were analysed with a probit model using package 'drc' (Ritz et al., 2016) in RStudio Version 1.2.133 (R Team 2020). In the limit tests, mortality in treatment groups was compared to control treatments using Fisher's exact test for small samples.

Results indicate that males and females are roughly equally sensitive to sulfoxaflor and to azoxystrobin in its formulation Amistar at 48 h post-exposure, when accounting for dose per bee. We note that males are significantly smaller than females (T-test, $t = 25.302$, $p<0.001$; Fig. 25), being on average 51% lighter than females. There is a tendency for males to be less sensitive to sulfoxaflor and azoxystrobin, when looking at dose per gram bodyweight, however this difference is not significant (Table 9). We note that mortality in the azoxystrobin oral treatment may not be attributed to the active ingredient alone, as other formulants in the commercial product may play a role in the mortality outcome.

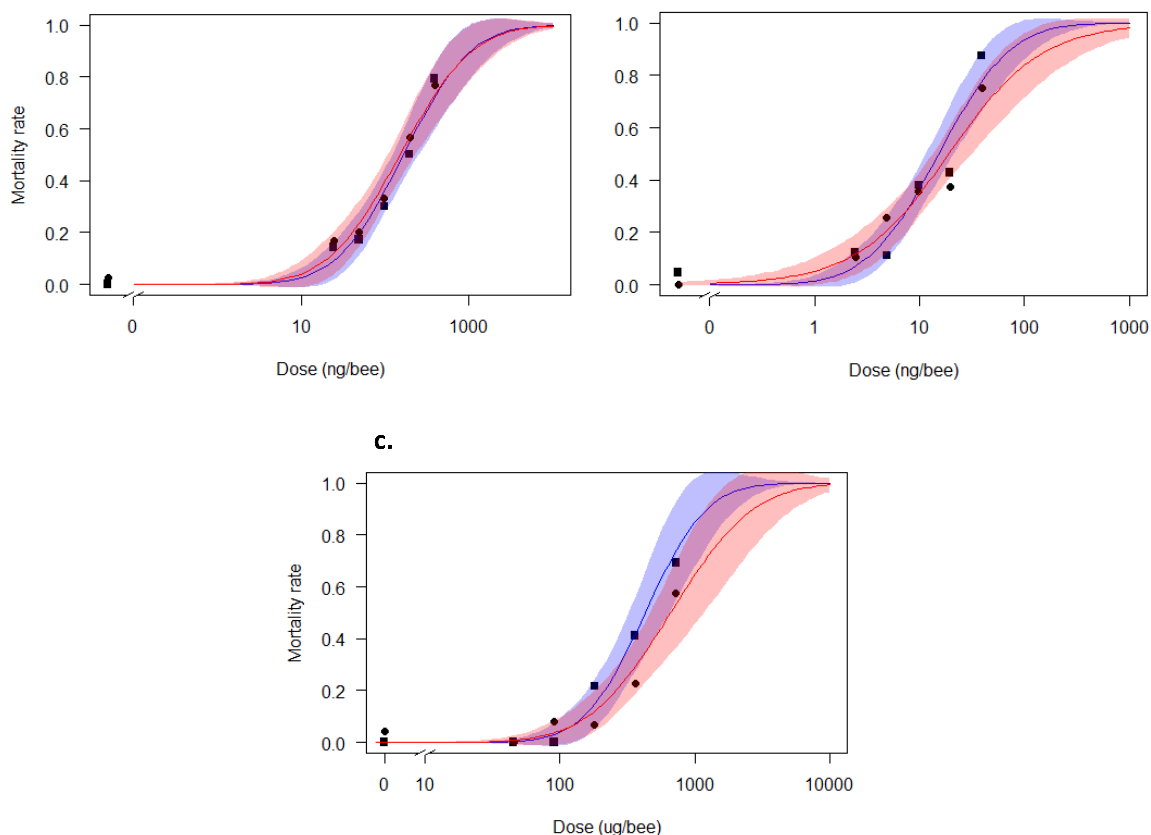

Figure 25. LD_{50} values of a. sulfoxaflor contact, b. sulfoxaflor oral, c. azoxystrobin oral. Males = blue squares, Females = red circles. Shaded area is 95% CI.

Table 9. LD_{50} values for *O. bicornis* males and females oral and contact exposure in ng/bee and ng/g bodyweight (95% CI).

	Route	Sex	n/treatment	LD50 (C.I.)	
				dose / bee	dose /g bee
Sulfoxaflor	Oral	Female	32-26	19.26 (12.8 - 28.99) ng	215.7 (143.4 - 324.6) ng
		Male	32-28	16.74 (12.67 - 22.13) ng	362.6 (274.4 - 479.2) ng
	Topical	Female	30	150.7 (105.6 - 215.2) ng	1702.8 (1192.8 - 2430.8) ng
		Male	30	145.1 (103.6 - 203.3) ng	3367 (2403.9 - 4716) ng
Azoxystrobin	Oral	Female	31-26	664.6 (427.9-1032) µg	7440 (4791-11556) µg
		Male	30-27	430.1 (287.1-573) µg	9314(6215-12409) µg
	Topical	Female	30	>100 µg	NA
		Male	30	>100 µg	NA
Glyphosate	Oral	Female	31-26	>100 µg	NA
		Male	30-26	>100 µg	NA
	Topical	Female	30	>100 µg	NA
		Male	30	>100 µg	NA

3. Assessment of chronic effects

3.1. Honey bee (*Apis mellifera*)

Lena Barascou, Deborah Sene, Yves Le Conte, Cedric Alaux (INRAE)

3.1.1. Mortality endpoints

3.1.1.1. Workers

Newly-emerged bees were sampled from 8 colonies and mixed. They were then placed in plastic cages (30 bees per cage). They were provided *ad libitum* with candy ((Apifonda® + powdered sugar) for 2 days and then exposed for 10 days to one of the pesticides (active substance: sulfoxaflor, azoxystrobin, glyphosate) or pesticide mixtures via contaminated sugar syrup (50% w/v sucrose, 0.1% acetone). Control groups were fed with pesticide-free sugar solution (50 % w/v sucrose, 0.1 % acetone).
We exposed worker bees to concentrations that were considered to be field realistic and a higher concentration representing a worst-case exposure scenario since LD_5 values were not yet available for worker bees at the time of experiment.

The pesticide concentrations were:

- Sulfoxaflor (S1 : 0.01, S2 : 0.1, S3 : 1 mg/kg)
- Glyphosate (G1 : 5, G2 : 30 mg/kg)
- Azoxystrobin (A1 : 0.2, A2 : 2 mg/kg).

We also exposed groups of bees to ternary pesticide mixtures as follows:

- M1: 0.1 mg/kg sulfoxaflor, 5 mg/kg glyphosate, 0.2 mg/kg azoxystrobin
- M2: 0.1 mg/kg sulfoxaflor, 30 mg/kg glyphosate, 2 mg/kg azoxystrobin
- M3 : 0.01 mg/kg sulfoxaflor, 5 mg/kg glyphosate, 0.2 mg/kg azoxystrobin
- M4 : 0.01 mg/kg sulfoxaflor, 30 mg/kg glyphosate, 2 mg/kg azoxystrobin

Dead bees were counted daily and removed until day 33. The highest and lowest concentrations of sulfoxaflor (S1 and S3) significantly decreased bee survival compared to control groups (Cox model, p<0.001; Fig. 26). No effect of glyphosate was observed, and only the lowest concentration of azoxystrobin reduced bee survival (A1). The mixtures M1 and M2, including the medium concentration of sulfoxaflor, did not affect bee survival but M3 and M4, including the lowest sulfoxaflor concentration, did.

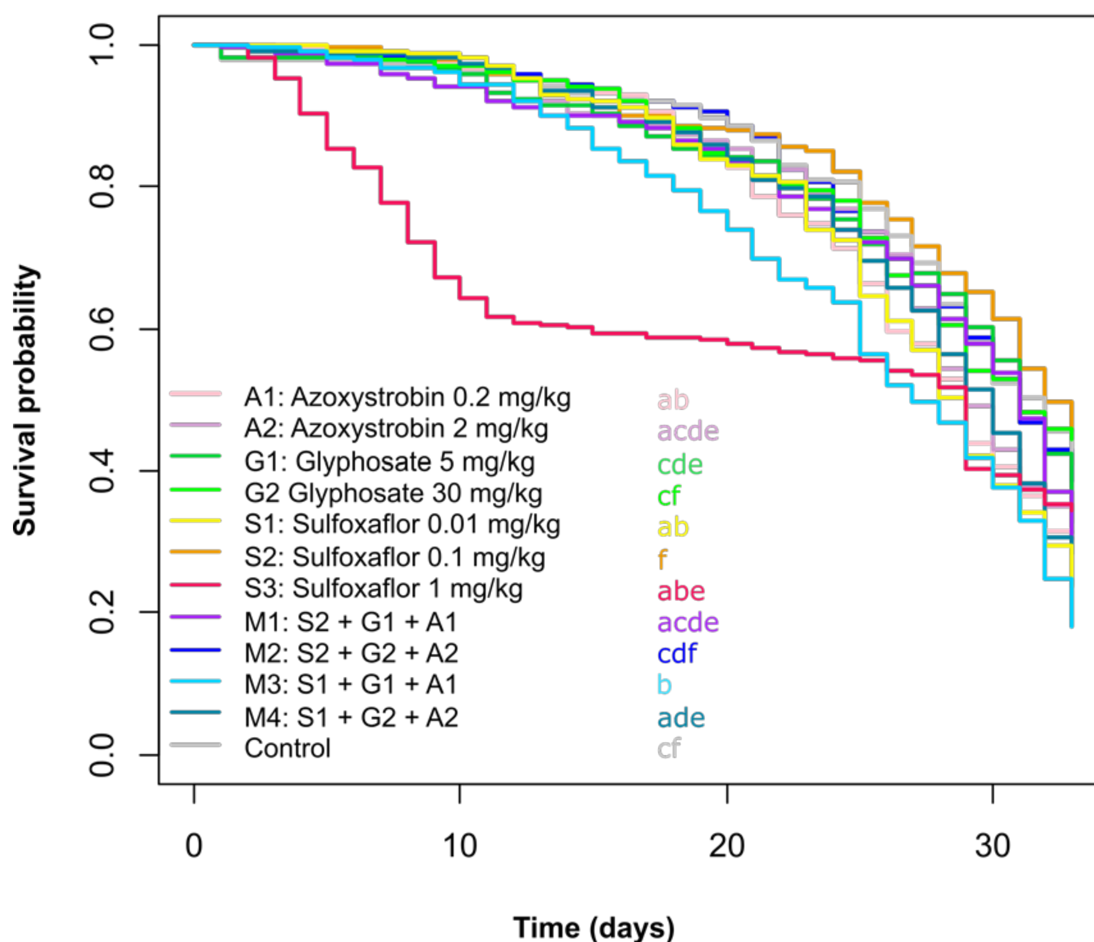

Figure 26. Effects of pesticides (alone or in combination) on honey bee worker survival. Data show survival probability over 33 days (n=8 cages of 30 bees per treatment). Different letters indicate significant differences between treatments (Cox model).

We then calculated the hazard ratio, which is defined as the ratio between the instantaneous risk in the treatment group and the risk in the control group occurring at a given time interval (in this case, day). We found that the highest risk of death was caused by the exposure to the mixture M3, followed by sulfoxaflor (S1 and S3) and azoxystrobin (A1), then by mixture M4 and azoxystrobin (A2) (Fig. 27). The others pesticide treatments did not cause any increase in the risk of death of worker bees.

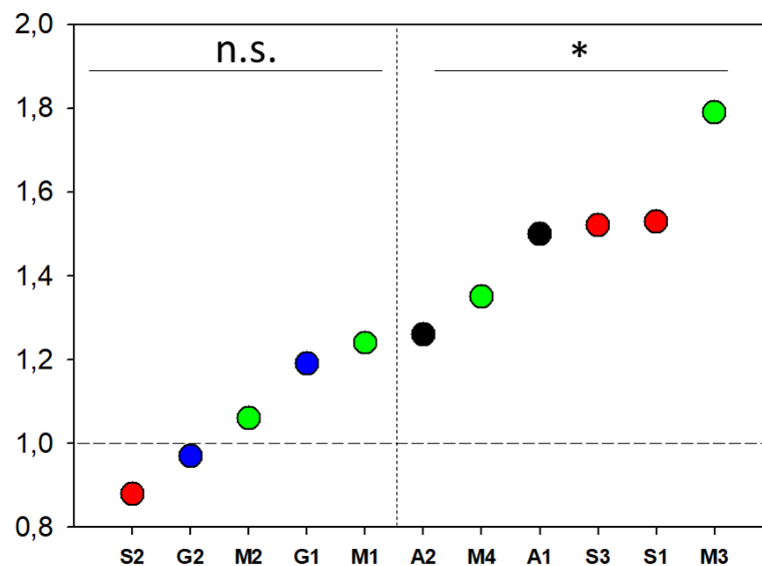

Figure 27. Hazard ratio for bees exposed to sulfoxaflor (S, red), azoxystrobin (A, black), glyphosate (G, blue) or the ternary mixtures (M, green). Asterisks indicate statistically significant risks of death caused by the pesticide (Cox model: $p < 0.05$) and the dotted line represents a hazard ratio of 1 (no mortality risk). The corresponding pesticide concentrations can be found above in the text.

Interestingly, our results showed a Non-Monotonic Dose-Response (NMDR) to sulfoxaflor. Indeed, the toxicity of sulfoxaflor did not increase gradually along the concentration gradient, but was only significant at the lowest and highest concentrations (the medium concentrations having no effect on bee mortality). The higher toxicity of the low sulfoxaflor concentration (0.01 mg/kg) compared to the medium concentration (0.1 mg/kg) was further confirmed by the higher toxicity of mixtures M3 and M4 (composed of S1: 0.01 mg/kg sulfoxaflor) than mixtures M1 and M2 (composed of S2: 0.1 mg/kg sulfoxaflor). Therefore, our results suggest that regulatory tests should address NMDRs by testing a large range of concentrations, especially low ones, to fully inform pesticide risk assessment.
Finally, we did not find any interaction effects between pesticides since the decrease in the survival rate caused by mixtures M3 and M4 did not differ from the decrease in survival rate caused by pesticide treatments S1 and A1 (Fig 26).

3.1.1.2. Queens

Each virgin queen (Buckfast origin) was reared in a plastic cage with the 4-5 workers bees that attended queen emergence and 20 newly-emerged bees. They were provided *ad libitum* with candy (Apifonda® + powdered sugar) for 2 days and then exposed for 10 days to one of the pesticides (active substance: sulfoxaflor, azoxystrobin, glyphosate) via contaminated sugar syrup (50% w/v sucrose, 0.1% acetone). We exposed workers bees to concentrations that were considered to be field realistic and a higher concentration representing a worst-case exposure scenario (see above). We notably tested pesticide concentrations that significantly elevated the hazard ratio in the worker mortality assays (see above):

- Sulfoxaflor (0.01 and 1 mg/kg) – S1 and S3
- Glyphosate (5 mg/kg) – G1
- Azoxystrobin (0.2 mg/kg) – A1

The survival of a total of 10 queens per experimental group was recorded for 10 days. After the chronic exposure, haemolymph was sampled from both queens and workers that were still alive for proteomics analysis (WP9). This will enable us to compare physiological responses to pesticides between queens and workers.

None of the virgin queens died over the 10 days of exposure to pesticides. This may be explained by the fact that queens were not directly exposed to pesticides. Indeed, they are normally fed royal jelly by worker bees, and extremely low quantities of pesticides reach the worker hypopharyngeal glands, where royal jelly is produced (Böhme et al., 2019). However, a significant decrease in worker bee survival was observed upon exposure to both concentration of sulfoxaflor (Fig. 28), confirming the previous mortality assays performed on worker bees (see above).

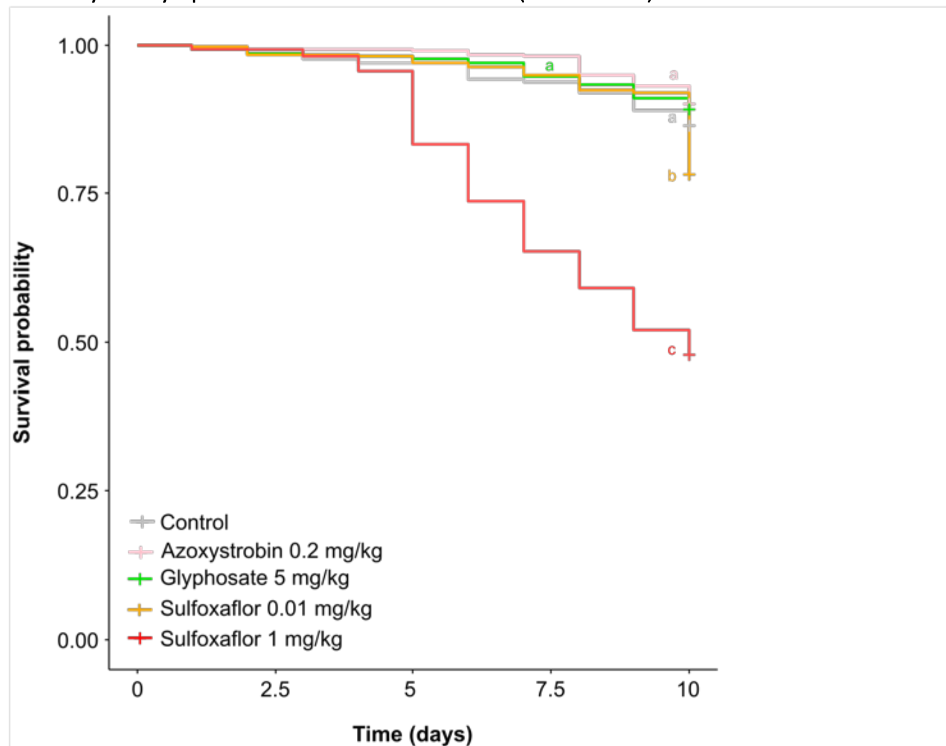

Figure 28. Effects of pesticides on the survival of worker bees reared with queens. Data show survival probability over 10 days (n=10 cages of 20 bees per treatment). Different letters indicate significant differences between treatments (Cox model, sulfoxaflor 0.01 mg/kg: $p=0.019$ and sulfoxaflor 1 mg/kg: $p<0.001$).

3.1.1.3. Drones

Experiments will be performed in May - June 2022 by BERN.

3.1.2. Behavioural endpoints

Pesticide risk-assessment guidelines for honey bees generally require determining the acute toxicity of a chemical over the short-term through fixed-duration tests. However, potential chronic or delayed effects resulting from an acute exposure (e.g. a single dose) are often overlooked, although the modification of a developmental process may have life-long consequences. To investigate this question, we exposed young honey bee workers to a single field-realistic dose of sulfoxaflor at the moment when they initiated orientation flights (preceding foraging activity). Newly-emerged bees were collected and marked with a data-matrix barcode glued on the thorax. They were then released into a colony equipped with an automated life-long monitoring devices (bee counter) recording their

flight activity: direction (in or out of the hive) and time of activity. At day 7, bees were individually fed with 2 µL of a solution of 30% (w/v) sucrose, 0.1 % acetone and sulfoxaflor at 5 µg/ml or 25 µg/ml, which corresponded to a theoretic exposure of 10 and 50 ng of sulfoxaflor/bee and to the ~LD_{50}/15 and LD_{50}/3 reported by EFSA for in-hive (i.e. young) bees (146 ng/bee) (EFSA 2014a). The exact concentrations were checked with LC-MS/MS (European Standard EN 15662:2018 procedure) and resulted in 8 µg/ml and 30 µg/ml for the prepared sulfoxaflor concentrations, which corresponded to a real exposure of 16 ng/bee and 60 ng/bee, respectively. We then tracked their flight activity and lifespan. The experimental procedure was repeated five times using 3 different colonies.

Among the 1108 tracked bees, we obtained data for 407 control bees, and 376 and 325 bees exposed to 16 and 60 ng of sulfoxaflor, respectively. Both doses of sulfoxaflor administered reduced the total number of flights (GLMM, $p<0.01$ for both doses) but did not affect bee survival (Cox model, 16 ng: $p=0.56$ and 60 ng: $p=0.19$; Fig. 29) or flight duration (GLMM, 16 ng: $p=0.96$ and 60 ng: $p=0.83$; Fig. 30). When looking at the time series of flight activity, effects were not immediate but delayed until the beginning of foraging activity, which was determined by using the *aof* R-package (Requier et al., 2020) (Fig. 30). Consequently, the number of foraging flights was lower in sulfoxaflor-exposed bees (24.75 ± 27.93 and 21.62 ± 22.29, respectively for bees exposed to 16 or 60 ng of sulfoxaflor) compared to control bees (32.60 ± 51.19; GLMM, dose 16 ng: $p < 0.01$ and dose 60 ng: $p < 0.005$; Fig. 30). This represented 24 and 33 % less foraging flights for the 16 and 60 ng doses, respectively.

The results therefore blur the general assumption in honey bee toxicology that acute exposure results in immediate and rapid effects and call for long-term recording and/or time-to-effect measurements, even upon exposure to a single dose of pesticide.

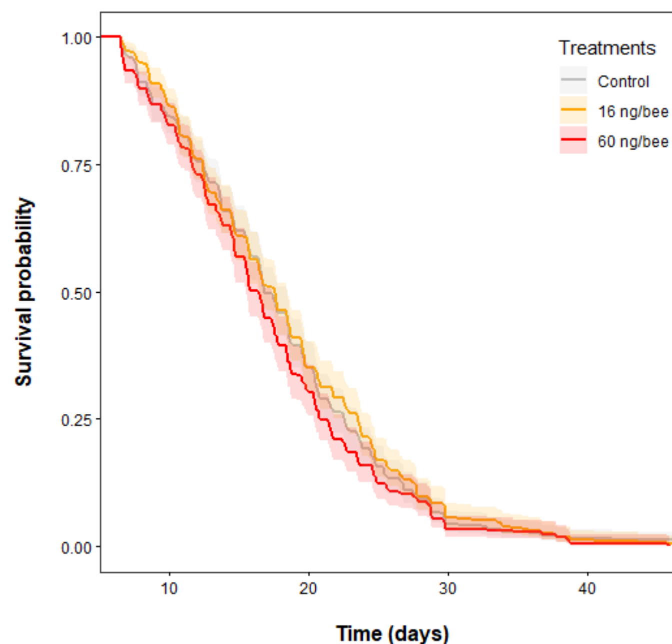

Figure 29. Survival probability of bees exposed to pesticide treatments. Data represent the survival probabilities of bees from day 7 to 45 with a 95% confidence interval.

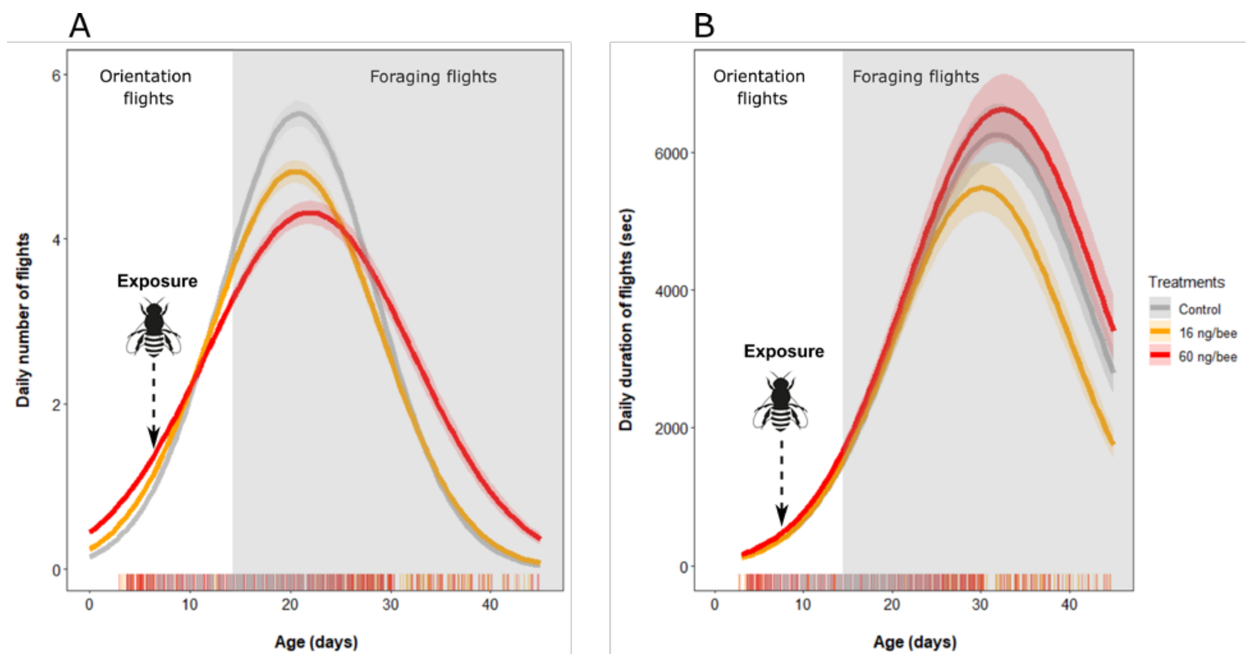

Figure 30. Time series of flight activity in response to pesticide treatments. (A) Number and (B) duration of daily flights (sec.) over bee lifetime (n = 407 control bees, n = 376 and 325 bees exposed to 16 or 60 ng of sulfoxaflor, respectively). Thick lines represent the model predictions with shaded areas indicating ½ standard error and the dashed arrows represent the day of exposure to sulfoxaflor. The transition to foraging activity started on average at 14.5 days. Coloured marks show the distribution of raw data along the horizontal axis.

The results were recently published: Barascou L., Requier F., Sene D., Crauser D., Le Conte Y., Alaux C. (2022) Delayed effects of a single dose of a neurotoxic pesticide (sulfoxaflor) on honey bee foraging activity. Science of the Total Environment, 805: 150351.

3.2. Bumble bee (Bombus terrestris)

Alberto Linguadoca, Harry Siviter, Morgan Morrison, Antoine Gekière, Ferozah Mahmood, Edward A Straw and Mark JF Brown (RHUL)

3.2.1. Workers

3.2.1.1. Survival, fertility and fecundity in microcolonies

Sulfoxaflor exposure reduces egg-laying in bumble bees (Bombus terrestris audax)

Results published in Siviter et al. (2020b) showed that a field realistic dietary exposure of 5 ppb sulfoxaflor over 15 days reduced egg-laying (Fig 31), but did not alter survival, ovarian development, or larval production in bumble bee (*Bombus terrestris audax*) microcolonies.

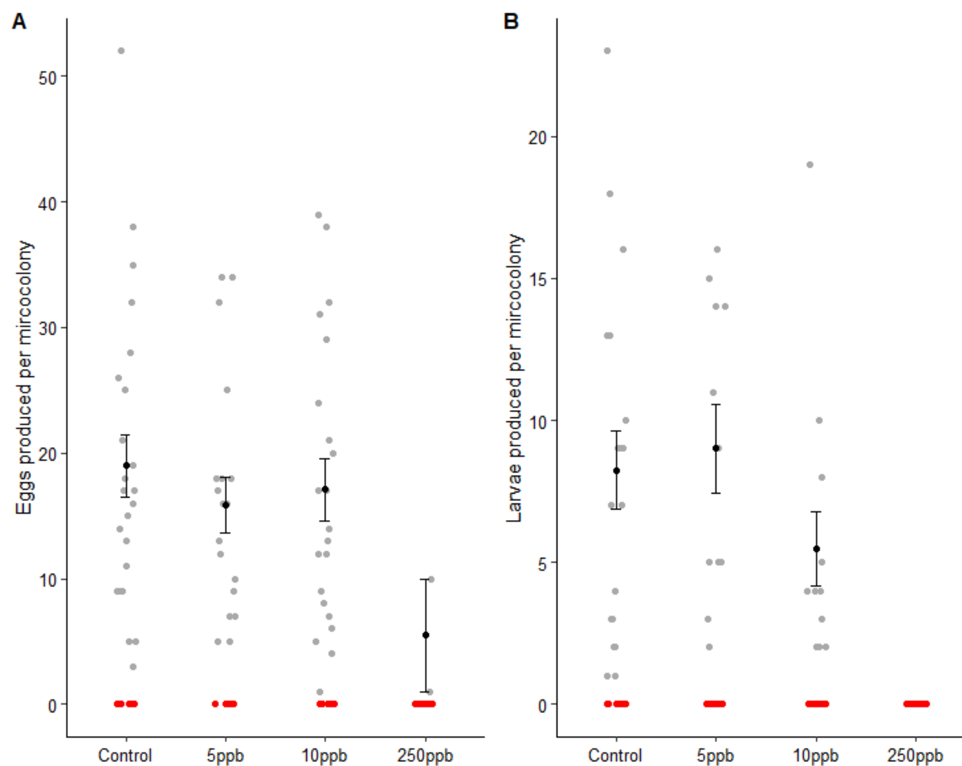

Figure 31: The mean (± SE) number of eggs (a) and larvae (b) produced per microcolony. Red dots show microcolonies that did not produce (a) eggs or (b) larvae. Grey dots show (a) egg counts, and (b) larval counts per microcolony. Standard errors are calculated from the non-zero data

Sulfoxaflor and nutritional deficiency synergistically reduce survival and fecundity in bumble bees
In this experiment we used a novel microcolony design, to investigate co-occurring, lethal and sublethal risks of sulfoxaflor and a dietary stress for bumble bees (*Bombus terrestris audax*). Using this new paradigm, we tested a worst-and best-case sulfoxaflor exposure scenario, mimicking pesticide dissipation of in nectar. Specifically, pesticide exposure was tested with a high and low sugar diet (hereby defined as nutritional limitation) in a fully factorial design, to test for possible synergisms of these two stressors.

Worst-case sulfoxaflor exposure caused sharp lethal effects, which were further exacerbated by the nutritional limitation. The best-case regime did not affect mortality. However, when the same pesticide exposure was paired with the nutritional limitation, sulfoxaflor significantly reduced the likelihood of bee survival (Fig. 32). The best-case sulfoxaflor exposure caused a reduction of food consumption, egg-laying and larval production. Like for mortality, the nutritional limitation additively or synergistically exacerbated effects on fecundity. Poor nectar quality exacerbated sulfoxaflor risks by simultaneously inducing physiological stress and increasing pesticide intake.

Our data show that non-mitigated label uses of sulfoxaflor may have major, yet severely neglected effects on bumble bee health, which may be exacerbated by nutritional stress.

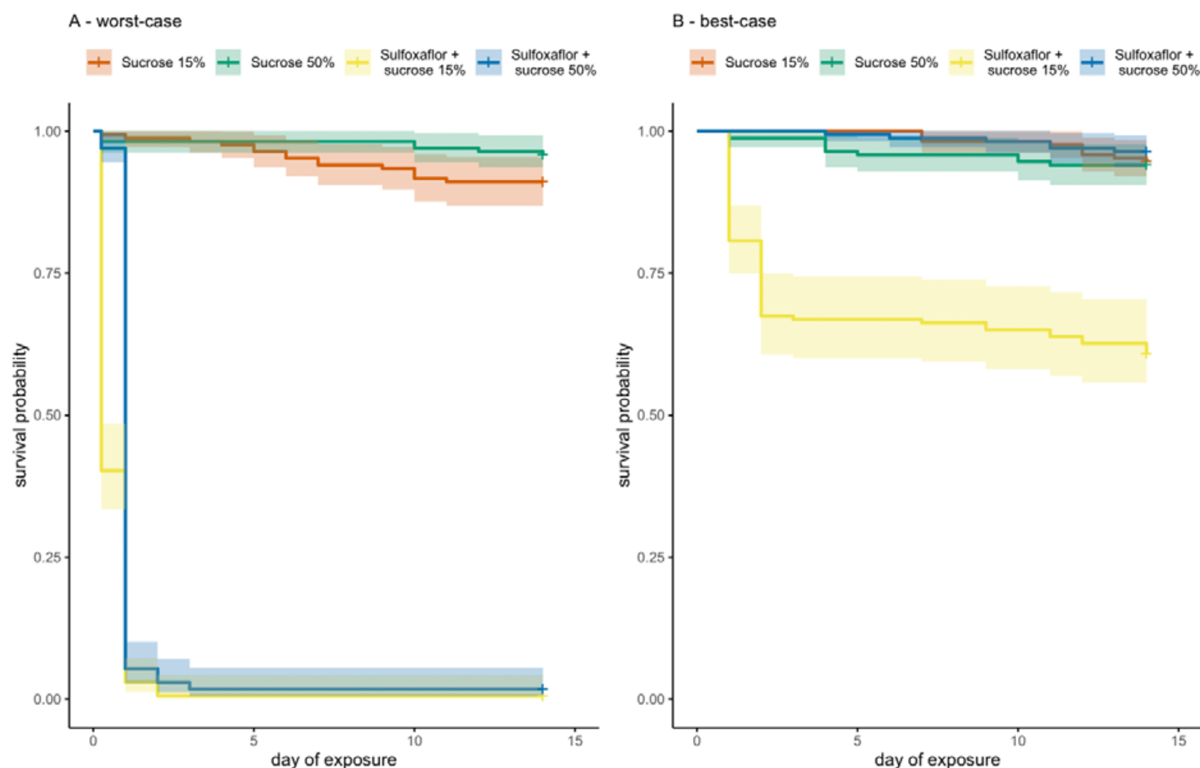

Figure 32. The Kaplan-Meier curves (± 95% CI) for the cumulative probability of survival over time of bumble bee workers under worst- (A) and best-case (B) exposure conditions

The effects of cyantraniliprole on survival, fecundity and activity levels in bumble bee (B. terrestris audax) microcolonies

Addressing Task 3.3, we tested whether a field-realistic exposure to cyantraniliprole may affect food consumption, mortality, fecundity and behaviour (i.e., activity levels) of workers housed in microcolonies. This novel insecticide, acting as a ryanodine receptor modulator and used as a seed and soil treatment, is currently being heavily studied.

Using the probabilistic exposure assessment described in Willis Chan et al. (2019) on a large published residue dataset (EFSA 2014b), we justified the selection of a conservative, field realistic concentration of 100 ppb cyantraniliprole. Queen-less microcolonies were chronically exposed to this field realistic concentration of cyantraniliprole through pollen and syrup for one week.

Mortality and fecundity were tested and statistically analysed using the methods described in Linguadoca et al. (2021) and Siviter et al. (2020a). Activity levels were defined as the proportion of time bees engaged in active behaviours, such as nursing, walking, grooming or gathering food. These behaviours were assessed by means of video recordings, which were later analysed using a widely used event-logging freeware software (Friard and Gamba, 2016). No effects were observed on short-term survival, fecundity or behaviour of bumble bee workers.

3.2.1.2. Olfactory learning

Addressing both Tasks 3.3 and 6.3, we used a Proboscis Extension Reflex (PER) paradigm in a fully crossed design to test the hypothesis that chronic exposure to the azoxystrobin and the trypanosomatid gut parasite *Crithidia bombi* may individually and/or interactively affect learning abilities in bumble bee (*Bombus terrestris audax*) workers. We used queenright commercial colonies (Agralan, UK), which, upon arrival, were divided into microcolonies. At this stage all workers were individually marked and randomly allocated to 4 microcolony boxes. Each microcolony was assigned

to one of the treatments (i.e., an untreated control, *C. bombi*, azoxystrobin and *C. bombi* + azoxystrobin). Upon housing and according to the treatment allocation, bees were fed a provision of sucrose syrup, spiked or not with a purified *inoculum* of 10 000 cells/bee. On day 6 of the experiment, bees belonging to the azoxystrobin or *C. bombi* + azoxystrobin groups were given a field-realistic concentration of 500 ppb a.i. for 4 consecutive days (Schatz and Wallner, 2009). All other bees were fed untreated syrup for the same timeframe. After exposure, marked bees were harnessed (Figure 9) and – on the following day - tested for PER as in Siviter et al. (2019) (Fig. 33).

We tested 175 bees belonging to 5 colonies in a staggered design. Seventy-three bees were excluded because they failed to respond to antennal stimulation with sucrose. The remaining 102 bees were tested for associative learning through PER (Siviter et al., 2019). We did not find evidence that azoxystrobin, *C. bombi* or their interaction affected responsiveness or learning in bumble bee workers.

Figure 33. Harnessed bumble bees before PER

3.2.2. Queens

Realistic soil exposure to cyantraniliprole did not affect hibernation success of bumble bee (B. terrestris audax) queens in the laboratory.

An unexplored, yet key aspect of pesticide risk assessment, is how agricultural soil contamination may affect the hibernation success of individual bumble bee queens. Here, we tested the hypothesis that long-term soil exposure to cyantraniliprole may reduce hibernation success in queens. Specifically, we designed a new laboratory test protocol, where individually housed bumble bee queens were exposed to artificial soil spiked at increasing field-realistic cyantraniliprole concentrations (Zhang et al., 2019) during hibernation. The test groups were untreated soil, 0.6 mg cyantraniliprole/kg dry soil and 1.85 mg cyantraniliprole/kg dry soil.

Gynes were mated in communal arenas, following the same methodology used in Baron et al. (2017), before being allocated by weight and colony of origin to the three treatments. Three days after mating, queens were individually housed in 50 ml centrifuge tubes filled with artificial soil (OECD 2016) spiked or not with cyantraniliprole (Fig. 34). To maximise contact, a hole was pre-dug in the soil prior to exposure. Upon housing, individually housed queens were hibernated in darkness at a constant temperature of 4 °C for 67 days. Upon hibernation, mortality was recorded, and live queens were weighed again to quantify their weight loss. To determine post-hibernation survival, live queens were housed in nicot cages and fed sucrose syrup for a period of 48h, over which mortality was recorded.

We found no evidence that soil cyantraniliprole exposure caused increased mortality (logistic regression, cyantraniliprole 1.85 mg/kg: $p = 0.7$, cyantraniliprole 0.6 mg/kg: $p = 0.8$) or bodyweight loss (glm, cyantraniliprole 1.85 mg/kg: $p = 0.2$, cyantraniliprole 0.6 mg/kg: $p = 0.3$).

Figure 34. The hibernation test unit. A 50ml centrifuge tube filled with artificial soil.

Effects of chronic, oral exposure to cyantraniliprole on hibernation and colony founding success of captive bumble bee queens (B. terrestris audax).

Newly emerged, aged-controlled bumble bee gynes were housed in single-sex, communal wooden boxes and fed untreated or pesticide spiked food (i.e., cyantraniliprole 100ppb through pollen and 30% sucrose: n Cyantraniliprole=110; n control=113) for 7 days (same exposure regime as for the microcolony experiment). During this phase, mortality and food consumption were recorded daily. Immediately after exposure, sexually mature gynes and males were mated in a custom-made 60cm×50cm×50cm wooden framed arena. Gynes were given 5 chances to mate – each lasting 40 minutes – before being discarded as unmated. Mating pairs were removed from the cage upon visual inspection and temporarily transferred into plastic boxes. Upon the end of copulation, males were removed from these boxes, while queens were kept in their cage till the next day, when their hibernation started. Mated queens were weighed and placed into 50ml Falcon tubes with ≃ 15mL damp sterilized sand and vent holes drilled in the lid. Tubes were kept vertically in darkness at a constant temperature of 4 °C for 12 weeks, during which queens were left undisturbed. At the end of the 12 weeks, mortality and bodyweight change were recoded.

Live queens were housed in custom-made acrylic boxes (width: 50 mm; length: 115 mm; height: 196 65mm) and kept at 26˚C and 60% humidity in full darkness. Each queen was given *ad libitum* access to fresh pollen and 30% w/w sucrose syrup.

We found evidence that oral exposure to Cyantraniliprole affected the long-term survival (Figure 35), but not colony initiation (Figure 36) of bumble bee queens.

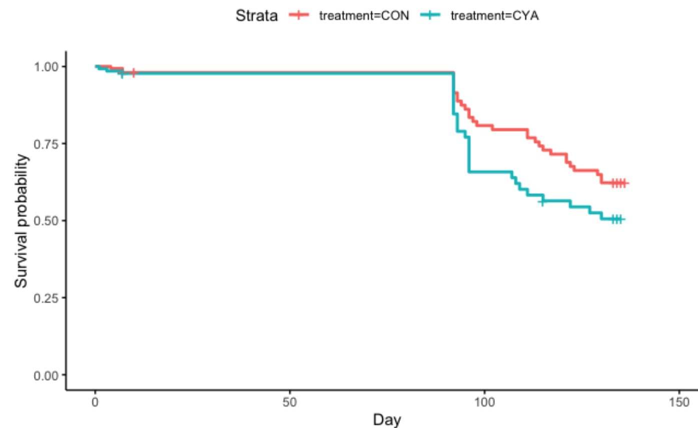

Figure 35. Long-term mortality assessment of queens. Kaplan-Meier mortality curves showing the likelihood of survival of bumble bee queens during exposure, hibernation and colony founding. Censored data are visually represented by vertical segments. CON=control; CYA=Cyantraniliprole.

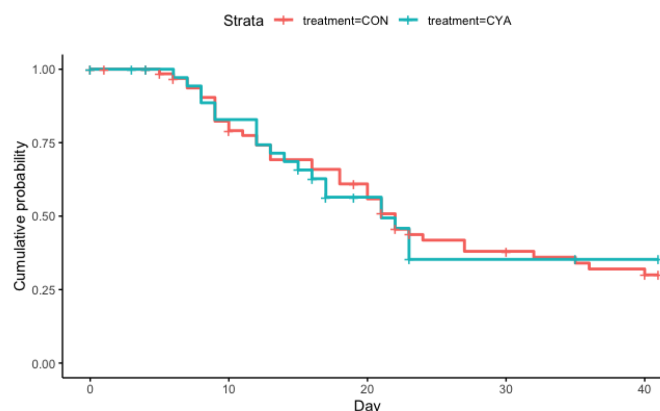

Figure 36. Proportion of queens that had produced eggs by each day after hibernation. Censored values (i.e., unsuccessful or dead queens) are visually represented by vertical segment. CON=control; CYA=Cyantraniliprole.

3.2.3. Males

We established whether a decaying, best-case field-realistic exposure regime to sulfoxaflor (Linguadoca et al., 2021) affected mating success and fertility in male bumble bees (*Bombus terrestris audax*). Exposure to sulfoxaflor was performed following the same procedure as in the cyantraniliprole experiments in queens (Baron et al., 2017). The effects of sulfoxaflor exposure on sperm viability was determined as in Minnameyer et al. (2021) (Fig. 37).

We did not find evidence that sulfoxaflor affected mating success or sperm viability in exposed males.

Figure 37. Bumble bee male genitalia before sperm extraction and viability assessment

3.3. Solitary bee (*Osmia bicornis*)

Sara Hellström, Robert Paxton (MLU)

3.3.1. Adults

Adult female *O. bicornis* were chronically exposed to field-realistic doses of the three PoshBee chemicals. Ten newly emerged females were placed in hoarding cages each provided with two sugar solution feeders. Cages were kept in daylight condition at room temperature for the 29-day duration of the test. The choice of literature-sourced, field-realistic concentrations of chemicals were preferred over the proposed LD_5 of the GA, since these data were not available at the start of the experiment. The dosing regime in this experiment was designed to mimic field-realistic exposure in the following way: an exponential model or quadratic model was fitted to a collection of residue time-series taken from the scientific and regulatory literature. A series of decreasing doses was extracted from these models, mimicking the natural breakdown of an active substance in crop nectar after field application. Based on these time-series, sugar solution containing the active ingredient was exchanged daily over four days (Table 10). During and after exposure, food consumption and mortality was monitored (Fig. 38). The resulting survival time series was analysed using a Cox proportional hazard model with Tukey post hoc test and Bonferroni correction. When comparing treatment model to a null model, the treatment model fit was significantly better (ANOVA: df = 3, $p<0.05$). The sulfoxaflor treatment stands out as being significantly different from the azoxystrobin and control treatments ($p<0.05$).

Table 10. Dosing regimes (mg/kg) and average dose consumed per bee across the four days based on food consumption/cage.

Treatment	day 1	day 2	day 3	day 4	Avg. dose/bee (ng)	N bees
Control	0	0	0	0	0	52
Sulfoxaflor	0.1	0.06	0.04	0.02	6	40
Azoxystrobin	1.2	0.8	0.5	0.2	82	30
Glyphosate	30	15	8	6	1270	49

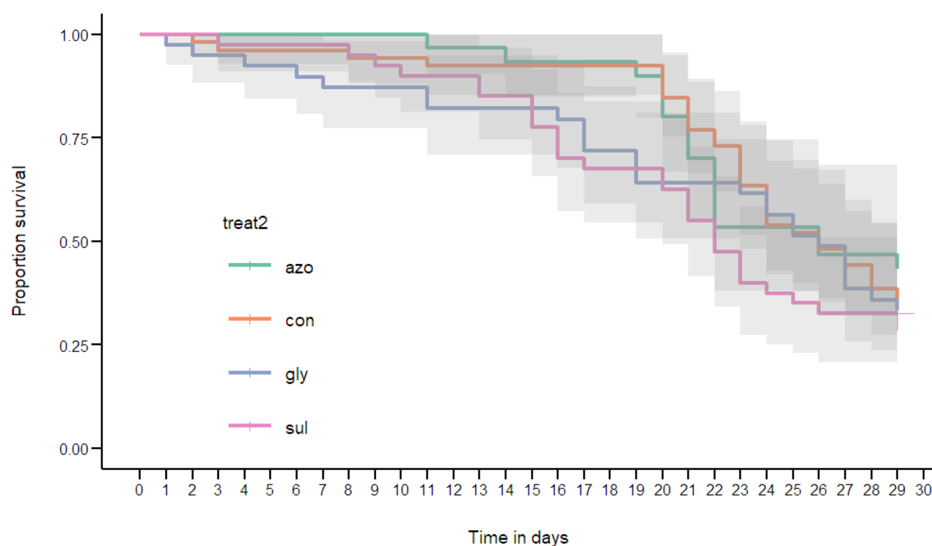

Figure 38. Survival curves of *O. bicornis* females exposed to decreasing pesticide doses from day 1-4. Shade area represent 95% confidence interval. Con: control, azo: azoxystrobin, gly: glyphosate, sul: sulfoxaflor

The total sulfoxaflor dose consumed by females amount to 31% of the LD_{50} as determined in this report. This did not cause acute mortality during the exposure period, but appears to have mildly influenced the long-term survival. We note that the exposure regime was an average of several residue analysis dataset, and the results may be different when testing the worst-case field-realistic scenario in a similar setup.

3.3.2. Larvae

Solitary bees can be chronically exposed to pesticides in different ways (Sgolastra et al., 2018). Contrary to the eusocial species where food is processed by worker bees prior to larval consumption, solitary bees normally develop directly on a pollen/nectar provision provided by the mother. The chronic toxicity of chemicals can thus be assessed by letting larvae develop on contaminated pollen provisions. This type of toxicological assay for *Osmia* sp. has recently been described in detail by Claus et al. (2021), and an adapted version of this protocol was used here. Entire nests (n=62) of *Osmia bicornis* were collected from a local population in a largely pesticide-free environment, in order to include both males and females. The newly laid eggs were removed from their maternal provision, randomized and transferred to artificial pollen provisions (350 mg/bee) made of organic, honey bee-collected pollen (Prunus mix, Abeilles Heureuses, France). Pollen provisions were contaminated with field-realistic concentrations of the three PoshBee chemicals as well as flupyradifurone, along with a negative control, a solvent control and a maternal provision control (Table 11). Flupyradifurone was included, since it is another relatively novel systemic insecticide which is currently being heavily studied. Larvae developed in closed 48-well cell culture plates (Nucon) at 21°C constant temperature and 40-60% relative humidity. Hatching rate and survival until cocoon spinning were recorded by checking the developmental stage of each larva every other day. Development time from egg hatching to cocoon spinning was determined (but not analysed in this endpoint). Cocoons were overwintered outside (September 2020 - March 2021) and allowed to emerge in controlled conditions during March and April 2021.

Table 11. Treatment descriptions, total larvae included in test, total hatched per sex and survival percentage.

Treatment	Concentration a.i (mg/kg)	Pollen	N hatched larvae	N female emerged	N male emerged	total survived	Percent survival
Sulfoxaflor	0.5	*Prunus* mix	59	38	16	54	91.5%
Azoxystrobin	0.012	*Prunus* mix	50	27	18	45	90.0%
Flupyradifurone	2.2	*Prunus* mix	38	16	14	30	78.9%
Glyphosate	20	*Prunus* mix	62	29	28	57	91.9%
Control	NA	*Prunus* mix	37	21	11	32	86.5%
Control Acetone	NA	*Prunus* mix	32	16	12	28	87.5%
Control Maternal pollen	NA	Maternal provision	41	17	7	24	58.5%

Cocoons were incubated at 15 degrees for nine days, after which temperature was raised to 21°C in order to induce emergence. Hatching success and time to emergence were assessed. Analysis was done in R and graphic elements were created using package ‘ggplot2’.

Emergence time was measured in days from incubation start to emergence using a linear mixed model with poisson distribution. Since *O. bicornis* is a protandrous species, males emerge before females (Figure 39). When sex was included as an explanatory variable, treatment had no effect on emergence time (GLM: df = 264, p>0.3).

Eggs which failed to hatch were not included in the analysis, since death from mechanical damage during moving as opposed to treatment could not be ruled out. Survival was thus assessed between hatching (1st larval instar) to cocoon spinning (5th larval instar) and over hibernation until successful emergence. Survival between treatments were analysed using generalized linear model and chi-square test. The only treatment to significantly differ from control was the maternal provision treatment (GLM: df = 1, p<0.0001). This may be due to fungal or other pathogens present in the non-sterilized pollen collected directly from in-field nests. When this treatment was removed, overall treatment effect was not significant (GLM: df = 4, p>0.3) (Figure 40).

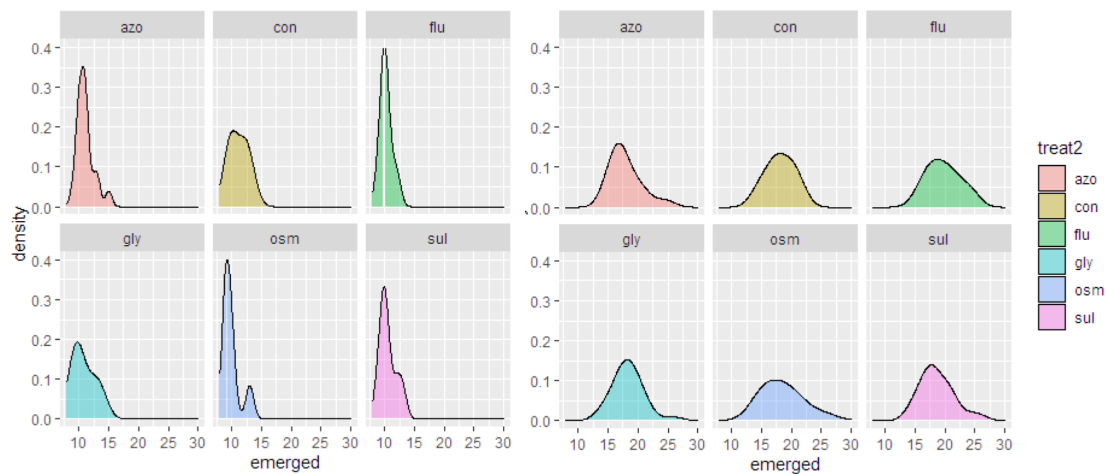

Figure 39. Density plot showing emergence time in days since incubation start for each treatment and males (left) and females (right). Con: control, azo: azoxystrobin, gly: glyphosate, sul: sulfoxaflor, flu: flupyradifurone, osm: control maternal pollen (maternal provision treatment)

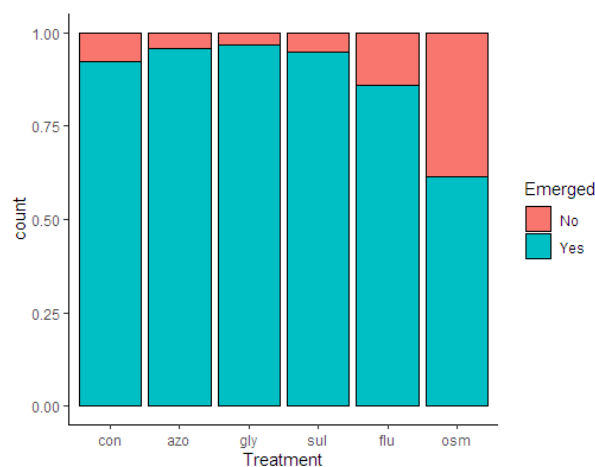

Figure 40. Stacked barplot showing survival from hatching to emergence for all treatments and sexes. Only maternal provision treatment ("osm") significantly differed from control.

We note that the amount of consumed active ingredient per larva cannot be reliably assessed, due to breakdown dynamics during the course of the experiment. Average time from hatching (when pollen consumption starts) to right before cocoon spinning (when consumption ceases) was on average 19.7 days, during which the active ingredients have time to break down significantly.

4. Assessment of interaction effects among pesticide classes

4.1. Honey bee (*Apis mellifera*)

Experiments on worker bees will be performed in the summer 2022 by CREA.

4.2. Bumble bee (Bombus terrestris)

Margret Jurison, Marika Mand (EMU), Alberto Linguadoca, Harry Siviter, Morgan Morrison, Antoine Gekière, Ferozah Mahmood, Edward A Straw and Mark JF Brown (RHUL)

4.2.1. Oral toxicity

Addressing Task 3.4, we characterised the combined toxicity of sulfoxaflor and azoxystrobin through oral acute exposure in bumble bee workers. We used a potentiation design (Azpiazu et al., 2021), where sulfoxaflor was tested at increasing doses, with or without a non-lethal dose of azoxystrobin, to test for synergisms between the two chemicals (Table 12). We selected a worst-case, field realistic concentration of azoxystrobin in nectar (1.45 mg a.s./l, Schatz and Wallner, 2009) to which we applied a multiplication factor of 10, to cover for all possible environmental variation (Spurgeon et al., 2016).

Table 12. Treatments in the bumble bee acute oral toxicity test

	Test item	N (initial)	N (after exclusion of non-feeders)	Mean mass mg ±SE	Treatment dose per bee (µg a.s./bee)
Workers	Sulfoxaflor	29-31	26-30	239 (5)	0.01, 0.04, 0.07, 0.1, 0.13, 0.16
Workers	Sulfoxaflor + azoxystrobin	30	26-30	241 (2)	sulf: 0.01, 0.04, 0.07, 0.1, 0.13, 0.16 azo: 0.58

We found no evidence of synergistic interaction of sulfoxaflor and azoxystrobin after acute oral exposure (Figure 41 and Table 13).

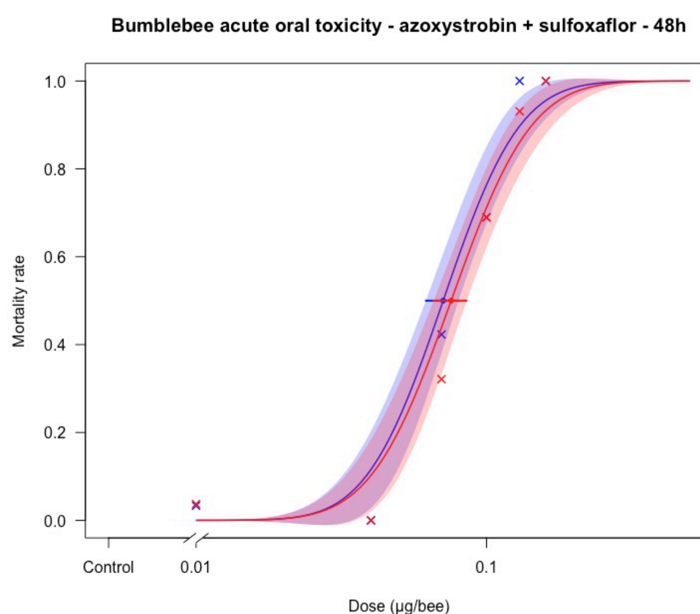

Figure 41. Acute oral toxicity of sulfoxaflor, alone (red) or combined with azoxystrobin (blue) in bumble bee workers. The 2-parameter log normal dose-response is described by a sigmoidal curve, along with the 95% confidence band (shaded area). The x marks indicate observed mortality levels at

each tested concentration 48 hrs post-exposure. The LD_{50} and 95% CI are described by points and horizontal segments respectively. Both pesticides were administered as active ingredients.

Table 13. Acute oral LD_{50} (95% CI) of sulfoxaflor, alone or in combination with a non-lethal dose of azoxystrobin.

Assessment time	Test item	Workers (µg/bee)
48 h	sulfoxaflor	0.076 (0.66 – 0.085)
48 h	Sulfoxaflor + azoxystrobin	0.071 (0.062 – 0.08)

4.2.2. Contact toxicity

The combined effects of sulfoxaflor, azoxystrobin and glyphosate on the survival of bumble bee (*Bombus terrestris* L.) workers was evaluated using contact exposure. The test methodologies were developed based on the OECD guidelines (OECD 2017). The methods used in these experiments have been described and published under Deliverable D3.2. Test species *B. terrestris* was purchased from the local supplier A.M.OZOLI (Cīruļdārzi, Eimuri, LV 2164 Latvia). Bumble bee workers were purchased as boxed queen-right colonies. Bumble bees were taken from 8 different colonies. All the bumble bees were randomly selected and evenly distributed by weight (avoiding extremely small and large sized workers). After selection, bees were individually allocated to a Nicot® cage and acclimatised to the test conditions (25 ± 1 °C, ~60% relative humidity and darkness) for 24 h with access to *ad libitum* and untreated 50 % weight per volume (w/v) aqueous sucrose solution.

Each experiment consisted of (1) undosed control (acetone), (2) positive control (dimethoate), (3) different dilutions of sulfoxaflor, (4) mixture of pesticides (sulfoxaflor x azoxystrobin and sulfoxaflor x azoxystrobin x glyphosate) and (5) one limit test dose of azoxystrobin or glyphosate (Table 14). Mortality data were recorded at 6, 24, 48, 72, 96 hours after treatments. Bees were considered as dead when they did not move their legs or antennae and did not respond to provocation.

Table 14. Concentrations and doses tested on *B. terrestris* workers. Number and mean mass of individuals are also shown.

N	Mean mass mg	Sulfoxaflor [mg/L]	Sulfoxaflor (µg/bee)	Azoxystrobin concentration [mg/L]	Azoxystrobin (µg/bee)	Glyphosate (µg/bee)
40	261	25000,12500, 5000, 2500, 500, 50	50,25,10,5,1,0.1	50000	100	100

Combined effects of sulfoxaflor and azoxystrobin on bumble bee B. terrestris survival

This study showed that acetone control and azoxystrobin did not increase mortality in bumble bees and survival remained at 100% during the experiment (Figure 42). As a positive control, however, dimethoate increased bumble bee mortality to 100% within 24 hours.

The active ingredient sulfoxaflor significantly reduced bumble bee worker survival at the highest doses (5, 10, 25 and 50 µg/bee), but not at the two lowest doses (0.1 and 1 µg/bee) (Figure 42). Interestingly, when combined with azoxystrobin, only the 25 and 50 µg/bee doses of sulfoxaflor caused a significant reduction of bee survival, suggesting antagonistic effects between the two pesticides at the 5 and 10 µg/bee doses of sulfoxaflor. This later trend was confirmed by co-exposure to sulfoxaflor at 25 µg/bee and azoxystrobin, which was less toxic than sulfoxaflor alone (25 µg/bee).

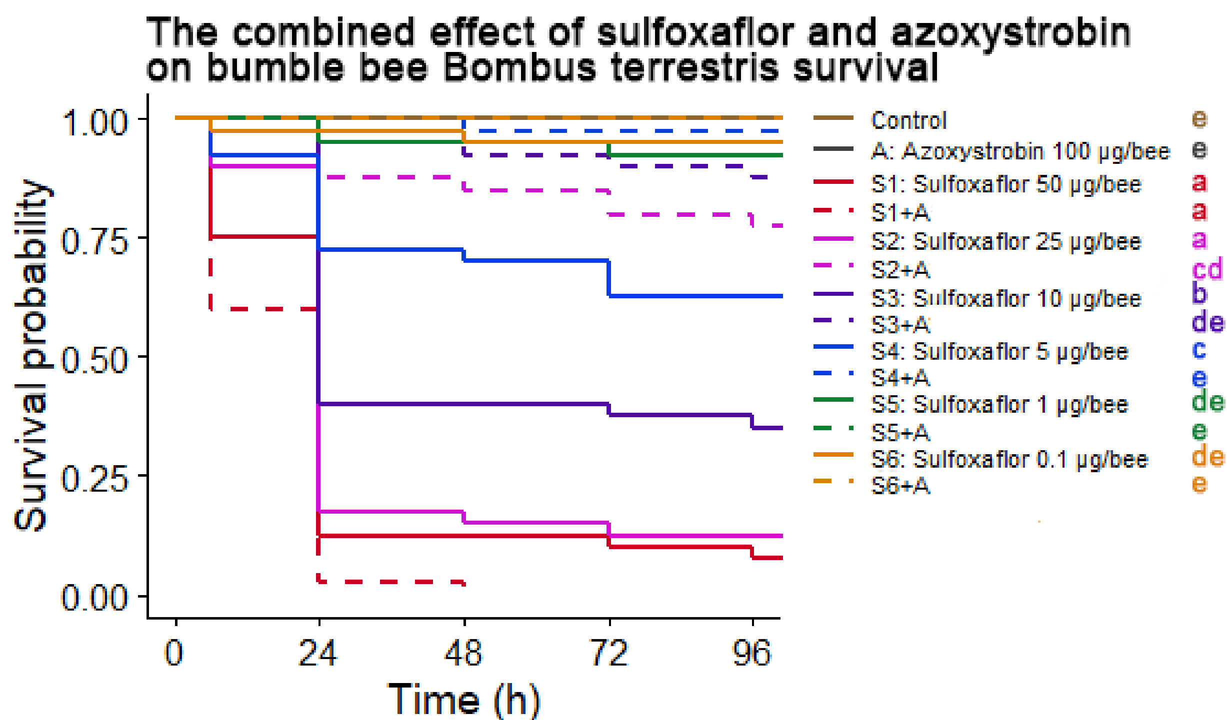

Figure 42. Combined effect of sulfoxaflor and azoxystrobin on bumble bee workers survival over 96 hours post-exposure. Different letters indicate significant differences between treatments (Kruskal-Wallis, p<0.01).

Combined effects of sulfoxaflor, azoxystrobin and glyphosate on bumble bee Bombus terrestris survival
Acetone control, azoxystrobin and glyphosate, did not cause any increase in bumble bee mortality (97.5-100%) (Figure 43). As a positive control, however, dimethoate increased bumble bee mortality to 100% within 24 hours.

The active ingredient sulfoxaflor significantly reduced bumle bee worker survival at the 10, 25 and 50 µg/bee doses; the 0.1, 1 and 5 µg/bee doses had no effect on bee survival 96 hours post-exposure (Figure 43). Co-exposure to azoxystrobin, glyphosate and sulfoxaflor at 0.1, 1 and 5 µg/bee did not increase bee mortality. The toxicity of sulfoxaflor at 25 and 50 µg/bee combined with azoxystrobin and glyphosate did not differ from the sulxaflor treatement at 25 and 50 µg/bee. Interestingly, the co-exposure to azoxystrobin, glyphosate and sulfoxaflor at 10 µg/bee did not affect bee mortality as compared to the control group, although sulfoxaflor at 10 µg/bee did increase bee mortality, suggesting again an antagonistic effect between the 3 pesticides.

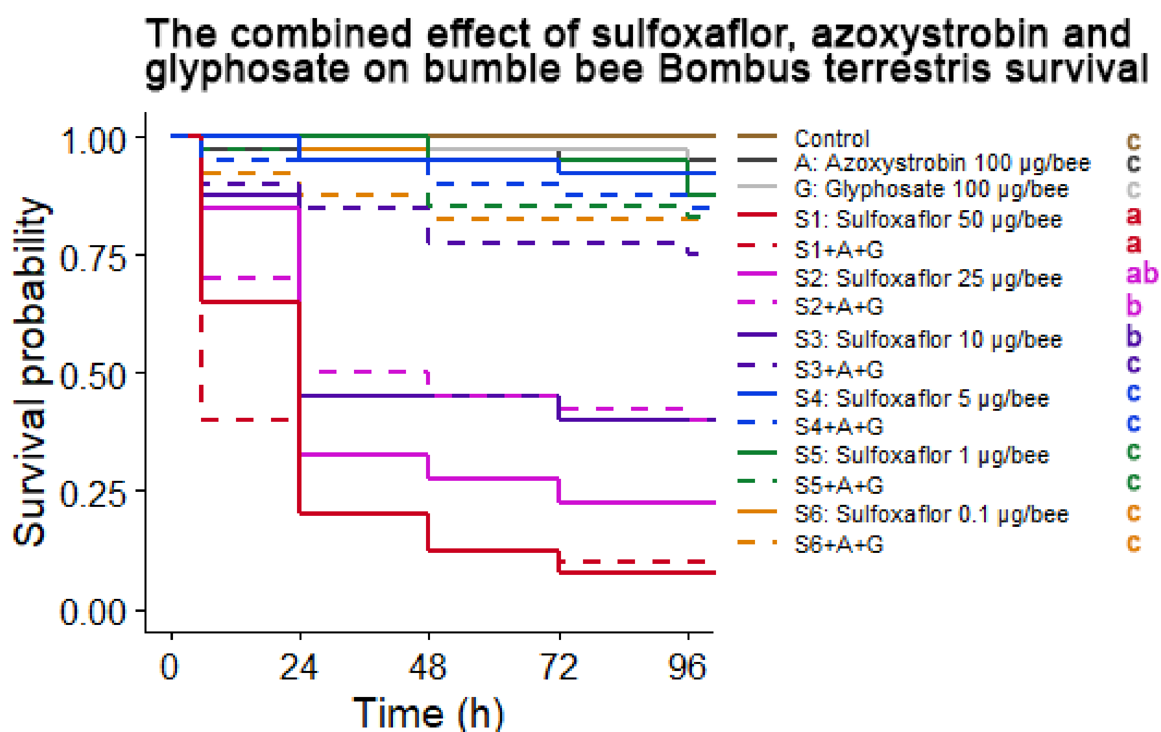

Figure 43. Combined effect of sulfoxaflor, azoxystrobin and glyphosate on bumble bee workers survival over 96 hours post-exposure. Different letters indicate significant differences between treatments (Kruskal-Wallis, p<0.01).

4.3. Solitary bee (*Osmia bicornis*)

We tested the dose-response of the butelonid insecticide flupyradifurone (pure active substance) in combination with azoxystrobin on female *O. bicornis.* A series of flupyradifurone doses, with or without a single, sub-lethal dose of azoxystrobin were administered orally using methods presented in Deliverable D3.2 (Table 15). The LD50 values were calculated using a log-normalised model and comparisons were performed in R using function "EDcomp" from package *drc.* We determined the oral LD50 of flupyradifurone to be 2.48 (CI 1.93 – 3.03) µg/bee at 48 hours post-exposure. For groups co-exposed to azoxystrobin, the LD50 value was determined to be 3.43 (CI 1.89 – 4.96) µg/bee (Figure. 44). Negative control and azoxystrobin-only controls had consistently low mortality (2.9-2.6%). Thus, there was no evidence of an interaction between the fungicide and flupyradifurone during oral exposure (Sensitivity ratio: 0.72, SE: 0.18, p = 0.13).

Table 15. Treatment description for the acute oral toxicity tests of flupyradifurone combined or not with azoxystrobin.

Sex	Test item	N (initial)	N (included)	Mean mass mg	Treatment dose per bee (µg a.s./bee)
Females	Flupyradifurone	38-21	18-30	129.1	9.1, 4.13, 1.88, 0.85, 0.425, 0.212
Females	Flupyradifurone + Azoxystrobin	41-21	16-30	128	flu: 4.13, 1.88, 0.85, 0.425, 0.212 azo: 2

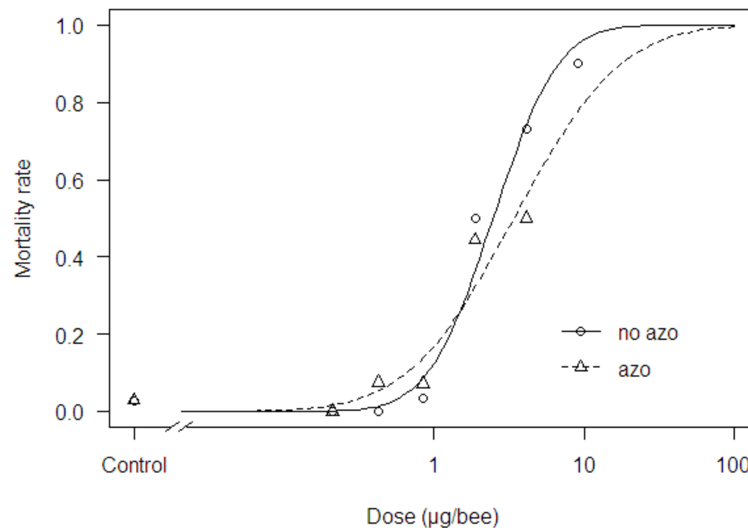

Figure 44. Acute oral toxicity of flupyradifurone combined or not with azoxystrobin in *O. bicornis* females at 48 hours post-exposure. The solid line represents flupyradifurone only and the dashed line flupyradifurone plus azoxystrobin.

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6. References

Azpiazu, C., Bosch, J., Bortolotti, L., Medrzycki, P., Teper, D., Molowny-Horas, R., Sgolastra, F., 2021. Toxicity of the insecticide sulfoxaflor alone and in combination with the fungicide fluxapyroxad in three bee species. Sci Rep-Uk 11, 6821.

Barascou L., Brunet J.-L., Belzunces L., Decourtye A., Henry M., Fourrier J., Le Conte Y., Alaux C. (2021) Pesticide risk assessment in honeybees: Toward the use of behavioral and reproductive performances as assessment endpoints. Chemosphere, 276: 130134

Baron, G.L., Jansen, V.A.A., Brown, M.J.F., Raine, N.E., 2017. Pesticide reduces bumblebee colony initiation and increases probability of population extinction. Nature Ecology & Evolution 1, 1308-1316.

Böhme, F., Bischoff, G., Zebitz, C., Rosenkranz, P., Wallner, K., 2019. From field to food II – will pesticide-contaminated pollen diet lead to a contamination of worker jelly? Journal of Apicultural Research 58, 542-549

Buss, D.S., McCaffery, A.R., Callaghan, A., 2002. Evidence for p-glycoprotein modification of insecticide toxicity in mosquitoes of the Culex pipiens complex. Med Vet Entomol 16, 218-222.

Chan, Q.W.T., Chan, M.Y., Logan, M., Fang, Y., Higo, H., Foster, L.J., 2013. Honey bee protein atlas at organ-level resolution. Genome Res 23, 1951-1960.

Claus, G., Pisman, M., Spanoghe, P., Smagghe, G., Eeraerts, M., 2021. Larval oral exposure to thiacloprid: Dose-response toxicity testing in solitary bees, Osmia spp. (Hymenoptera: Megachilidae). Ecotox Environ Safe 215, 112143.

Dahlgren, L., Johnson, R.M., Siegfried, B.D., Ellis, M.D., 2012. Comparative toxicity of acaricides to honey bee (Hymenoptera: Apidae) workers and queens. J Econ Entomol 105, 1895-1902.

EFSA, 2013. Guidance on the risk assessment of plant protection products on bees (Apis mellifera, Bombus spp. and solitary bees). EFSA Journal 11, 3295.

EFSA, 2014a. Conclusion on the peer review of the pesticide risk assessment of the active substance sulfoxaflor. EFSA Journal 12, 3692.

EFSA, 2014b. Conclusion on the peer review of the pesticide risk assessment of the active substance cyantraniliprole. EFSA Journal 12, 3814.

Evanno, G., Regnaut S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. Mol Ecol 14, 2611-2620.

Friard, O., Gamba, M., 2016. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods in Ecology and Evolution 7, 1325-1330.

Garnery, L., Solignac, M., Celebrano, G., Cornuet, J.M., 1993. A simple test using restricted PCR-amplified mitochondrial DNA to study the genetic structure ofApis mellifera L. Experientia 49, 1016-1021.

Henriques, D., Browne, K.A., Barnett, M.W., Parejo, M., Kryger, P., Freeman, T.C., Muñoz, I., Garnery, L., Highet, F., Jonhston, J.S., McCormack, G.P., Pinto, M.A., 2018a. High sample throughput genotyping for estimating C-lineage introgression in the dark honeybee: an accurate and cost-effective SNP-based tool. Sci Rep-Uk 8, 8552.

Henriques, D., Parejo, M., Vignal, A., Wragg, D., Wallberg, A., Webster, M.T., Pinto, M.A., 2018b. Developing reduced SNP assays from whole-genome sequence data to estimate introgression in an organism with complex genetic patterns, the Iberian honeybee (Apis mellifera iberiensis). Evol. Appl. 11, 1270-1282.

Johnson, R.M., 2015. Honey bee toxicology. Annu Rev Entomol 60, 415-434.

Ladas, A., 1972. The influence of some internal and external factors upon the insecticide resistance of honeybee. Apidologie 3, 55-78.

Laurino, D., Manino, A., Patetta, A., Porporato, M., 2013. Toxicity of neonicotinoid insecticides on different honey bee genotypes. B Insectol 66, 119-126.

Li, X., Schuler, M.A., Berenbaum, M.R., 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu Rev Entomol 52, 231-253.

Linguadoca, A., Rizzi, C., Villa, S., Brown, M.J.F., 2021. Sulfoxaflor and nutritional deficiency synergistically reduce survival and fecundity in bumblebees. Sci Total Environ 795, 148680.

Medrzycki, P., Giffard, H., Aupinel, P., Belzunces, L.P., Chauzat, M.P., Classen, C., Colin, M.E., Dupont, T., Girolami, V., Johnson, R., Le Conte, Y., Luckmann, J., Marzaro, M., Pistorius, J., Porrini, C., Schur, A., Sgolastra, F., Delso, N.S., van der Steen, J.J.M., Wallner, K., Alaux, C., Biron, D.G., Blot, N., Bogo, G., Brunet, J.L., Delbac, F., Diogon, M., El Alaoui, H., Provost, B., Tosi, S., Vidau, C., 2013. Standard methods for toxicology research in Apis mellifera. Journal of Apicultural Research 52, 60.

Meikle, W.G., Weiss, M., Stilwell, A.R., 2016. Monitoring colony phenology using within-day variability in continuous weight and temperature of honey bee hives. Apidologie 47, 1-14.

Milone, J.P., Rinkevich, F.D., McAfee, A., Foster, L.J. & Tarpy, D.R., 2020. Differences in larval pesticide tolerance and esterase activity across honey bee (*Apis mellifera*) stocks. *E*cotoxicoly Environ. Saf. 206**,** 111213.

Minnameyer, A., Strobl, V., Bruckner, S., Camenzind, D.W., Van Oystaeyen, A., Wäckers, F., Williams, G.R., Yañez, O., Neumann, P., Straub, L., 2021. Eusocial insect declines: Insecticide impairs sperm and feeding glands in bumblebees. Sci Total Environ 785, 146955.

OECD, 2013. Test No. 237: Honey Bee (Apis Mellifera) Larval Toxicity Test, Single Exposure.

OECD, 2016. Test No. 232: Collembolan Reproduction Test in Soil.

OECD, 2017. Test No. 247: Bumblebee, Acute Oral Toxicity Test.

Poquet, Y., Vidau, C., Alaux, C., 2016. Modulation of pesticide response in honeybees. Apidologie 47, 412-426.

Prado, A., Pioz, M., Vidau, C., Requier, F., Jury, M., Crauser, D., Brunet, J.-L., Le Conte, Y., Alaux, C., 2019. Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient honey bees. Sci Total Environ 650, 1250-1260.

R Team 2020. Studio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/.

Requier, F., Henry, M., Decourtye, A., Brun, F., Aupinel, P., Rebaudo, F., Bretagnolle, V., 2020. Measuring ontogenetic shifts in central-place foragers: A case study with honeybees. J Anim Ecol 89, 1860-1871.

Rinkevich, F.D., Margotta, J.W., Pittman, J.M., Danka, R.G., Tarver, M.R., Ottea, J.A., Healy, K.B., 2015. Genetics, Synergists, and Age Affect Insecticide Sensitivity of the Honey Bee, Apis mellifera. Plos One 10, e0139841.

Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2016. Dose-Response Analysis Using R. Plos One 10, e0146021.

Sandrock, C., Tanadini, M., Tanadini, L.G., Fauser-Misslin, A., Potts, S.G., Neumann, P., 2014. Impact of Chronic Neonicotinoid Exposure on Honeybee Colony Performance and Queen Supersedure. Plos One 9, e103592.

Schatz, F., Wallner, K., 2009. Pflanzenschutzmittelapplikation in blühenden raps (Brassica napus) und deren auswirkungen auf die rückstandssituation in honig, nektar und pollen der honigbiene (Apis mellifera L.). Universität Hohenheim.

Sgolastra, F., Hinarejos, S., Pitts-Singer, T.L., Boyle, N.K., Joseph, T., Lūckmann, J., Raine, N.E., Singh, R., Williams, N.M., Bosch, J., 2018. Pesticide Exposure Assessment Paradigm for Solitary Bees. Environ Entomol 48, 22-35.

Sgolastra, F., Medrzycki, P., Bortolotti, L., Maini, S., Porrini, C., Simon-Delso, N., Bosch, J., 2020. Bees and pesticide regulation: Lessons from the neonicotinoid experience. Biol Conserv 241, 108356.

Siviter, H., Bailes, E.J., Martin, C.D., Oliver, T.R., Koricheva, J., Leadbeater, E., Brown, M.J.F., 2021. Agrochemicals interact synergistically to increase bee mortality. Nature 596, 389-392.

Siviter, H., Folly, A.J., Brown, M.J.F., Leadbeater, E., 2020a. Individual and combined impacts of sulfoxaflor and <i>Nosema bombi</i> on bumblebee (<i>Bombus terrestris</i>) larval growth. Proceedings of the Royal Society B: Biological Sciences 287, 20200935.

Siviter, H., Horner, J., Brown, M.J.F., Leadbeater, E., 2020b. Sulfoxaflor exposure reduces egg laying in bumblebees Bombus terrestris. J Appl Ecol 57, 160-169.

Siviter, H., Scott, A., Pasquier, G., Pull, C.D., Brown, M.J.F., Leadbeater, E., 2019. No evidence for negative impacts of acute sulfoxaflor exposure on bee olfactory conditioning or working memory. PeerJ 7, e7208.

Spurgeon, D., Hesketh, H., Lahive, E., Svendsen, C., Baas, J., Robinson, A., Horton, A., Heard, M., 2016. Chronic oral lethal and sub-lethal toxicities of different binary mixtures of pesticides and contaminants in bees (Apis mellifera, Osmia bicornis and Bombus terrestris). EFSA Supporting Publications 13, 1076E.

Srinivas, R., Udikeri, S.S., Jayalakshmi, S.K., Sreeramulu, K., 2004. Identification of factors responsible for insecticide resistance in Helicoverpa armigera. Comp Biochem Physiol C Toxicol Pharmacol 137, 261-269.

Storck, V., Karpouzas, D.G., Martin-Laurent, F., 2017. Towards a better pesticide policy for the European Union. Sci Total Environ 575, 1027-1033.

Suchail, S., Guez, D., Belzunces, L.P., 2000. Characteristics of imidacloprid toxicity in two Apis mellifera subspecies. Environ Toxicol Chem 19, 1901-1905.

Thompson, H.M., Maus, C., 2007. The relevance of sublethal effects in honey bee testing for pesticide risk assessment. Pest Management Science 63, 1058-1061.

Tison, L., Holtz, S., Adeoye, A., Kalkan, Ö., Irmisch, N.S., Lehmann, N., Menzel, R., 2017. Effects of sublethal doses of thiacloprid and its formulation Calypso® on the learning and memory performance of honey bees. J Exp Biol 220, 3695-3705.

Willis Chan, D.S., Prosser, R.S., Rodríguez-Gil, J.L., Raine, N.E., 2019. Assessment of risk to hoary squash bees (Peponapis pruinosa) and other ground-nesting bees from systemic insecticides in agricultural soil. Sci Rep-Uk 9, 11870.

Woodcock, B.A., Bullock, J.M., Shore, R.F., Heard, M.S., Pereira, M.G., Redhead, J., Ridding, L., Dean, H., Sleep, D., Henrys, P., Peyton, J., Hulmes, S., Hulmes, L., Sárospataki, M., Saure, C., Edwards, M., Genersch, E., Knäbe, S., Pywell, R.F., 2017. Country-specific effects of neonicotinoid pesticides on honey bees and wild bees. Science 356, 1393-1395.

Zhang, Z., Xu, C., Ding, J., Zhao, Y., Lin, J., Liu, F., Mu, W., 2019. Cyantraniliprole seed treatment efficiency against Agrotis ipsilon (Lepidoptera: Noctuidae) and residue concentrations in corn plants and soil. Pest Management Science 75, 1464-1472.