# Synthesising the toxicokinetics and toxicodynamics of agrochemicals on bees

## Deliverable D3.4

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PoshBee

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

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## Summary

Within the PoshBee Project we have tested three bee species – honey bees *Apis mellifera*, bumble bees *Bombus terrestris* and solitary bees *Osmia bicornis* – for their sensitivity to pesticides and analysed the clearance of pesticides from bees. For each species, all castes and sexes were studied. We synthesised the mortality data (LD50 or results of limit tests) with the toxicokinetic patterns and analysed this against the background of inter- and intraspecific variation in life-histories of the tested bees.

The clearance of sulfoxaflor is relatively similar across all bee species tested and in females after contact treatment it tends to be retained. The toxicity increases over time independently of the clearance from the body. The clearance of azoxystrobin was rapid in *Osmia* and bumble bees, as well as in honey bee queens, but in honey bee workers there was very little clearance. Similar to sulfoxaflor the toxicity increased over time, although the residues were detected at very low levels. Glyphosate tended to be retained in bumble bees after contact treatment but cleared rapidly after oral treatment. For *Osmia* bees only in males after contact treatment was the glyphosate almost lost.

The toxicity of a pesticide is dependent on the exact dosage, but also the exposure route and time, as well as the speed of detoxification and clearance from a body. The assessment for the hazard that a less toxic pesticide might pose, can be largely dependent on the exposure route. The effects of pesticide toxicity can increase even after the molecules have been cleared out of the body.

## 1. Introduction

Environmental pollution is a major threat to many non-target organisms. Among pollutants, the pesticides used on fields threaten organisms living in or visiting the fields (Devine & Furlong, 2007). The economically and ecologically important bee-pollinators utilise flowering fields as their main food source and thus are exposed to pesticide residues found in fields (Alkassab & Kirchner, 2016; Belsky & Joshi, 2020; Chmiel et al., 2020; Ponce-Vejar et al., 2022). Understanding the survival of bees exposed to pesticides is a key aspect for environmental risk assessment.

To alleviate the risk from pesticides to bees, there are regulations that require toxicity tests for any new compound. Initially, honey bees were selected as model organisms to represent all pollinators, and consequently tests with worker honey bees have provided the predominant evidence-base for risk assessment of pollinators. More recently, however, it has been emphasised that these bees are not true representatives for the majority of bees (Topping et al., 2021). Since 2013 (EFSA, 2013) the inclusion of other bee species (bumble bees and solitary bees) and non-adult stages, as well as chronic and sublethal effects of pesticides on bee health, have been incorporated into risk assessments.

A full understanding of the effects of pesticides needs to include exposure dosages, times and time course of clearance of the molecules from the bee bodies. Synthetic molecules have different degradation and elimination times, which may depend on both abiotic and biotic factors. Common abiotic factors drive the degradation of molecules on surfaces, for example, solar radiation or temperature. Inside a body, the processes are more complicated. The molecules are adsorbed into the tissues, distributed all through the body, metabolised and excreted – these are toxicokinetic pathways which determine the chemical concentration inside the body (McCarty & Mackay, 1993). Toxicokinetic rates can be variable throughout individuals belonging to the same species (Spurgeon et

al., 2011) and are affected by behaviour, anatomical traits, metabolic capacity, stress-response capacity and microbiome differences (Spurgeon et al., 2020).

There is no clear understanding about relationships between toxicodynamic patterns and clearance rates in organisms. Earlier attempts to describe the relationship between toxicokinetic and -dynamic processes have been carried out largely on mammals (including humans) and aquatic organisms. For bees there are only a few first attempts to model these relationships. The models are based on generalised unified threshold model for survival (GUTS), which can be used to predict survival rate under untested exposure conditions (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2018). However, the outcomes have retained large uncertainty levels, and the results most probably depend on bee species with different physiological, morphological and behavioural traits (Baas et al., 2022). To our best knowledge, there are no studies comparing toxicokinetic-toxicodynamic assessments across and within bee species and chemicals from different classes. While morphological traits can affect toxicokinetic and/or toxicodynamic processes, there is also a lack of knowledge on differences between developmental stages and castes/sexes of bees.

The aim of the present research is to measure the degradation and elimination of chemicals from bee bodies and relate this information to the toxicodynamic measurements over three bee species *Apis mellifera*, *Bombus terrestris* and *Osmia bicornis*. We also broaden our study to different developmental stages, castes and sexes. The agrochemicals selected belong to three different classes: the relatively novel insecticide sulfoxaflor, a common fungicide azoxystrobin and the most used herbicide glyphosate. We discuss the results of oral and contact treatments and possible impacts of temporal clearance patterns on the biological meaning of these patterns from the perspective of accumulation and chronic exposure.

# 2. Materials and Methods

## 2.1. Honey bee *Apis mellifera*

Honey bee (*Apis mellifera*) workers, queens and drones were tested with sulfoxaflor, azoxystrobin and glyphosate in its commercial formulation RoundUp Platinum.

### 2.1.1. Toxicokinetics

For sulfoxaflor and azoxystrobin the substrates were prepared with a simplified QuEChERS method (Poshbee deliverable D3.1). For glyphosate analyses the samples were extracted in aqueous media and analysed by UPLC-MS/M. The comprehensive methods for bee treatments employed in the experiment are described in PoshBee deliverable D3.2. Samples weighing at least 2 g were used for chemical analyses. Bees were exposed orally and topically to sublethal doses of the chosen agrochemicals and sampled at different time points following the exposure.

Newly emerged *A. mellifera* worker bees (30 per cage) were used. For oral exposure, each cage received a feeder containing a total of 300 µL of test feeding solution (=10 µL per bee), for a duration of 4 hours (maximum). For the contact exposure, 1µl of the test solution was applied on the dorsal side of the thorax of each bee, with a micropipette. The test solutions contained respectively 5 ng of sulfoxaflor, 1µg of azoxystrobin, or 1µg of glyphosate.

Virgin queens were individually exposed to 5 ng of sulfoxaflor, 1 µg of azoxystrobin, or 1 µg of glyphosate. For oral exposure, queens were fed individually by hand with a micropipette (2 µl of a sugar solution laced with the pesticide). Topical exposure was performed on the queen thorax (2 µl of

acetone containing the pesticide). Honey bee queens were then sampled at 0, 6 and 24 hr post-exposure. For each pesticide, mode of exposure (oral, topical), and time post-exposure, a pool of 15 queens were stored at -20°C until pesticide residue analysis.

Drones were tested using the same methods and dosages (1 ng of sulfoxaflor, 1 µg of azoxystrobin and glyphosate) as for honey bee workers. In contact treatments, the initial levels were measured immediately after exposure, while final levels were measured at the end of the experiment. In oral treatments, there was a high mortality in the cages and it was not possible to collect sufficient individuals for the chemical analyses.

#### 2.1.2. Toxicodynamics

The contact and oral dose-responses for sulfoxaflor, azoxystrobin and glyphosate were determined in honey bee workers, queens and drones. The methods used in these experiments have been described and published under PoshBee Deliverable D3.2 and D3.3.

Honey bee workers (20 per cage) were fed with 200 µl of the test feeding solution and mortality was assessed at 6, 24 and 48 hours post-exposure. The $LD_{50}$ (D3.3) was calculated for sulfoxaflor as active pure substance. Both oral and contact toxicity of azoxystrobin and glyphosate was tested with the commercial formulations Amistar and Roundup Platinum, respectively. Limit tests with doses 100 µg/bee for both pesticides instead of $LD_{50}$ (see Deliverable D3.2) were used. The experiments with drones were carried out with the same dosages and time point assessments.

### 2.2. Bumble bee *Bombus terrestris*

Bumble bee colonies were purchased from local suppliers for toxicokinetic and toxicodynamic tests of the target chemicals sulfoxaflor, azoxystrobin and glyphosate (in formulations of RoundUp ProActive (oral) or RoundUp FLex (contact)) (see also Poshbee deliverable D3.1, Deliverable D3.2 and D3.3).

#### 2.2.1. Toxicokinetics

The same analytical techniques as with honey bees were used for pesticide residue assessments. For oral treatments, each worker bumble bee was treated with a no effect level dose (NOEL) of 0.08 µg/bee, queen bumble bees with 0.18 µg/bee and male bumble bees with 0.02 µg/bee of sulfoxaflor. All test materials were added to 40 µL 50% w/v sucrose solution. For contact treatment, each worker bumble bee was treated with NOEL of 1 µg, bumble bee queens with 20 µg/bee and male bumble bees with 0.1µg/bee of the sulfoxaflor. With Amistar in the oral treatment, the NOEL was 80 µg/bee for workers and males and 350 µg/bee for queen bumble bees. With glyphosate in the oral treatment a limit test dose 200 µg/bee (workers, queens) and 100 µg/bee (males) was used. In contact treatments workers and queens were administered with a limit test dose of 200 µg/bee, and males with a limit test dose of 100 µg/bee of Amistar and glyphosate (a.i. calculated from commercial formulation). Bumble bees were sampled up to 72h post-exposure.

#### 2.2.2. Toxicodynamics

The methods used in these experiments have been modified from OECD guidelines (OECD 2017) and are described and published under Deliverable D3.2. The experiments consisted of controls and a minimum of 5 increasing doses of Sulfoxaflor and Amistar, and one limit test dose of glyphosate (RoundUp ProActive or RoundUp FL). Mortality data were recorded up to 72 hours after treatment, except in the case of contact treatment with glyphosate 48h.

### 2.3. Solitary bee *Osmia bicornis*

Males and females from a commercially reared *Osmia bicornis* population were recruited to test the toxicokinetics and toxicodynamics of the target chemicals sulfoxaflor, azoxystrobin and glyphosate in its commercial formulation Roundup ProActive.

#### 2.3.1. Toxicokinetics

Oral treatment. The methods used for exposure and husbandry of the bees are described in detail in Deliverable D3.2. Modification from protocols described herein include group housing (n=9 individuals/cage) in metal cages (9*9*5 cm) as opposed to individual housing. The food source during testing was given *ad libitum* 50% w/v sucrose solution. The sulfoxaflor nominal dose given was 0.003 µg/bee, azoxystrobin 1 µg/bee, and for glyphosate 100 µg/bee. All doses were dissolved in 20 µL 50% w/v sucrose solution. The final time point was 48 hours from exposure for sulfoxaflor and 96 hours for azoxystrobin and glyphosate exposure.

Contact treatment. The nominal doses given were sulfoxaflor 0.00313 µg/bee (dissolved in acetone), azoxystrobin 1µg/bee (dissolved in acetone), and glyphosate (RoundUp ProActive, diluted in water and with 0.01% TritonX added as surfactant) given in a 1µL droplet. At least 1 g of bee bodies were collected per time point. The initial time point was immediately after exposure, while the final time point was 48 hours from exposure for sulfoxaflor and 8 days for azoxystrobin. For glyphosate, the final time point was 14 days post-exposure.

#### 2.3.2. Toxicodynamics

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. The experiments consisted of controls and a minimum of 5 increasing doses of Sulfoxaflor (oral and contact) and Amistar (oral), and one limit test dose of azoxystrobin (contact) and glyphosate (RoundUp ProActive) (oral and contact). Mortality data were recorded up to 48 hours after treatment.

## 3. Results

### 3.1. Honey bee *Apis mellifera*

#### 3.1.1. Sulfoxaflor

The initial clearance pattern after oral treatment is similar in worker and queen honey bees. After contact treatment, the clearance followed an almost linear pattern achieving 78% by 72h in workers (Figure 1, Table 1). In drones the clearance rate was lowest (58% by 96h). In queens there was no clearance of sulfoxaflor residues by 6h, however, by 24h the same level was achieved as in drones by 96h. The change in oral toxicity from 6h to 24 is higher in drones compared to queens. In drones, contact toxicity plateaued after 48h. In orally treated queens despite the fast degradation of sulfoxaflor, the toxicity continues to increase.

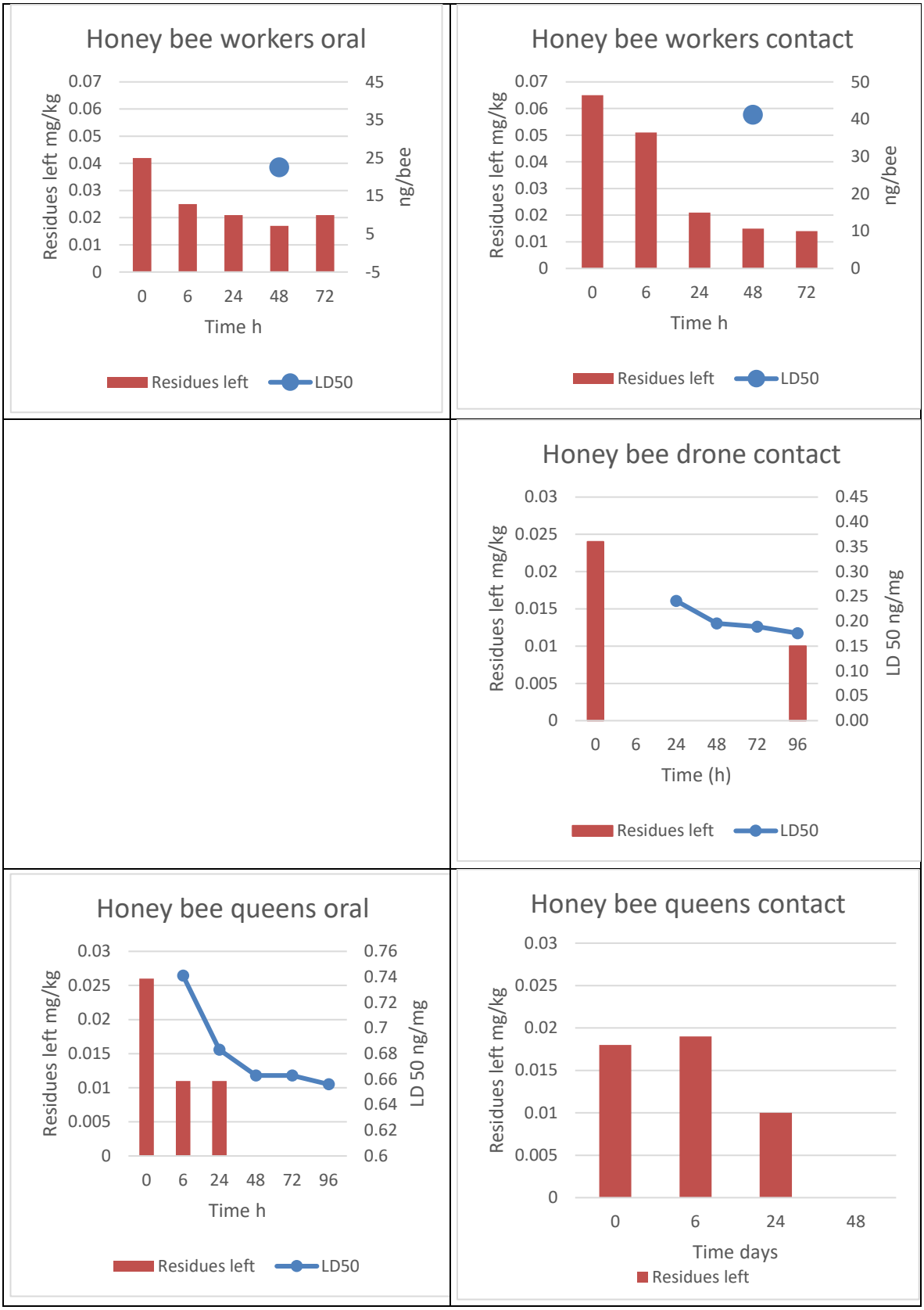


**Figure 1: Concentration of sulfoxaflor residues (mg/kg) in *Apis mellifera* workers, drones and queens after oral and contact exposure. Bees were sampled up to 96h after exposure.**

**Table 1: The clearance (%) of sulfoxaflor after oral and contact treatment in honey bee workers, queens and drones**

| Castes | Clearance % | |
|---|---|---|
| | Oral % | Contact % |
| Worker | 50 (72h) | 78 (72h) |
| Drone | - | 58 (96h) |
| Queen | 58 (24h) | 44 (24h) |

### 3.1.2. Amistar/Azoxystrobin

After oral treatment the azoxystrobin showed very little clearance in workers after 10 days, while in queens it was very rapid (Figure 2, Table 2). After contact treatment, the degradation was rapid in both workers and queens and degradation was clearly visible at the first time point post-application (Figure 2). The low degradation in workers is related also to higher mortality because of azoxystrobin.


**Figure 2: Concentration of azoxystrobin residues (mg/kg) and LD50/limit test in *Apis mellifera* workers and queens after oral (left) and contact exposure (right). Bees were sampled at 3 time points after exposure.**

**Table 2: The clearance (%) of azoxystrobin after oral and contact treatment in honey bee workers within 10 or 7 days, queens within 24 h**

| Castes | Clearance % | |
|---|---|---|
| | Oral % | Contact % |
| Worker | 15 (10days) | 87 (7days) |
| Queen | 92 | 81 |

### 3.1.3. Glyphosate

In contrast to sulfoxaflor and azoxystrobin, the clearance of glyphosate was higher in orally-treated queens and workers. After oral treatment in queens there was a linear decrease in glyphosate residues until the last time point (24 hr), but not so after contact treatment where there was an initial increase of the residues (Figure 3, Table 3). This initial increase in the queen bodies might be due to the progressive diffusion of glyphosate through the cuticle. After contact treatment we saw no degradation in queens, and only very low degradation in workers. In drones, after contact treatment the degradation appeared until 7 days, after which the level of residues plateaued.

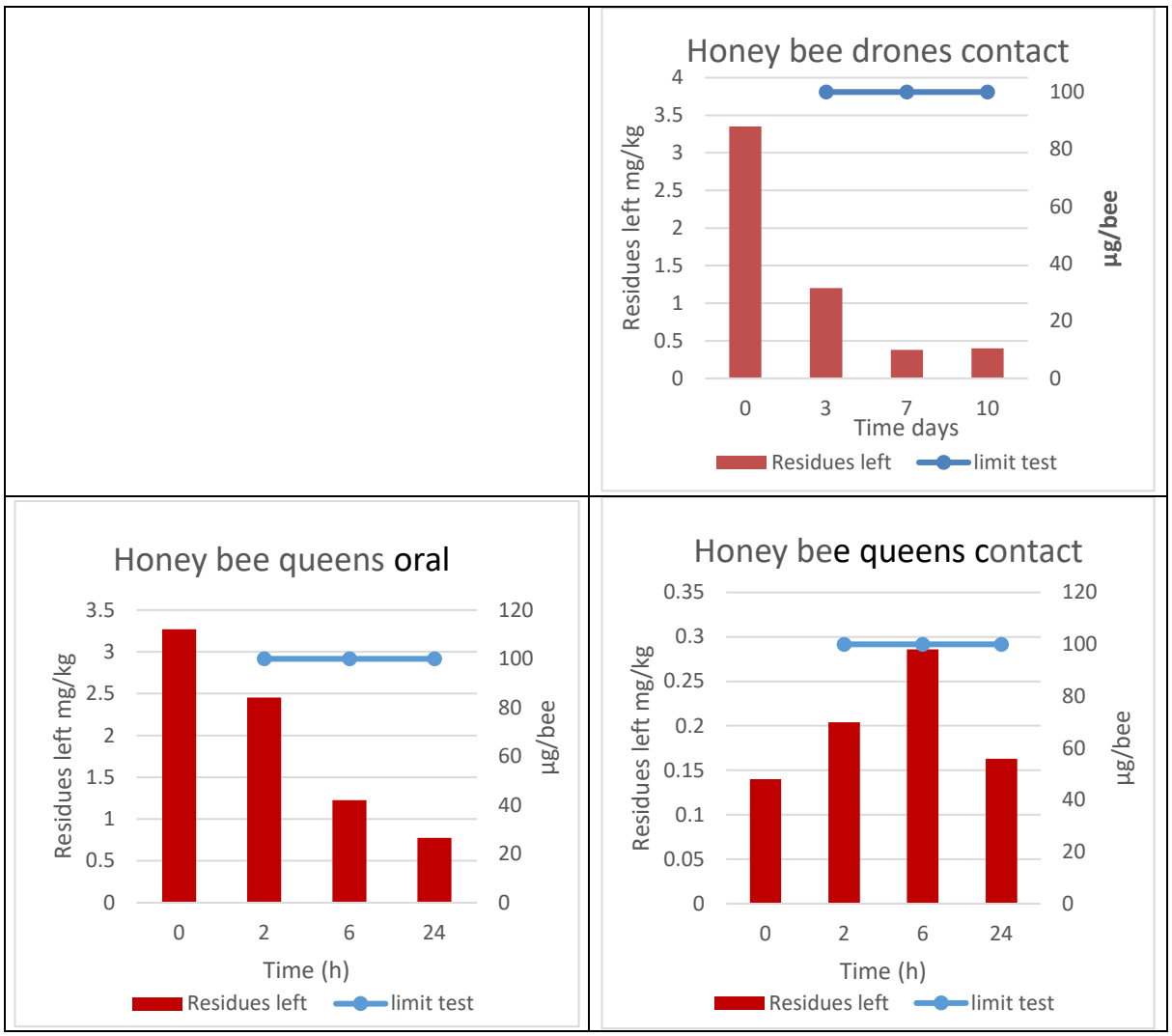


**Figure 3: Concentration of glyphosate residues (mg/kg) in *Apis mellifera* queens and drones after oral (left) and contact exposure (right). Bees were sampled at 4 time points after exposure.**

**Table 3: The clearance (%) of glyphosate after oral and contact treatment in honey bee queens and drones within 24 h**

| Castes | Clearance % | |
|---|---|---|
| | **Oral %** | **Contact %** |
| Drones | - | 88 |
| Queen | 76 | 0 |

## 3.2. Bumble bee *Bombus terrestris*

### 3.2.1. Sulfoxaflor

After oral treatment with sulfoxaflor in the bumble bee workers the residues stayed at the initial level at least for 6h and dropped rapidly by 24h (Figure 4, Table 4). In males the initial degradation was faster, but similarly to workers, by 24h the residue level had dropped drastically, and then plateaued. In queens, however, the clearance was much slower, the residue level was halved by 48h and clearance by 72h was almost complete.

In contrast, after contact treatment the absolute degradation was lower in all groups, and extremely low in queens (Figure 4, Table 4). Sulfoxaflor residues showed an almost linear decrease until the last time point (72h) in both workers and males, but in queens there was an initial increase of residues and no clear pattern of decline or increase after that.

The temporal patterns of sensitivity to sulfoxaflor are somewhat different between the three castes of bumble bees. In workers and males by both treatments the sensitivity pattern changes over time in parallel with the clearance patterns. However, in queens given contact treatment, the sulfoxaflor clearance is extremely slow without affecting the $LD_{50}$ pattern. In bumble bee workers, the high sensitivity to sulfoxaflor appears already with 6h while in males and queens the change in sensitivity between 6h and 24 or 48 h is larger.

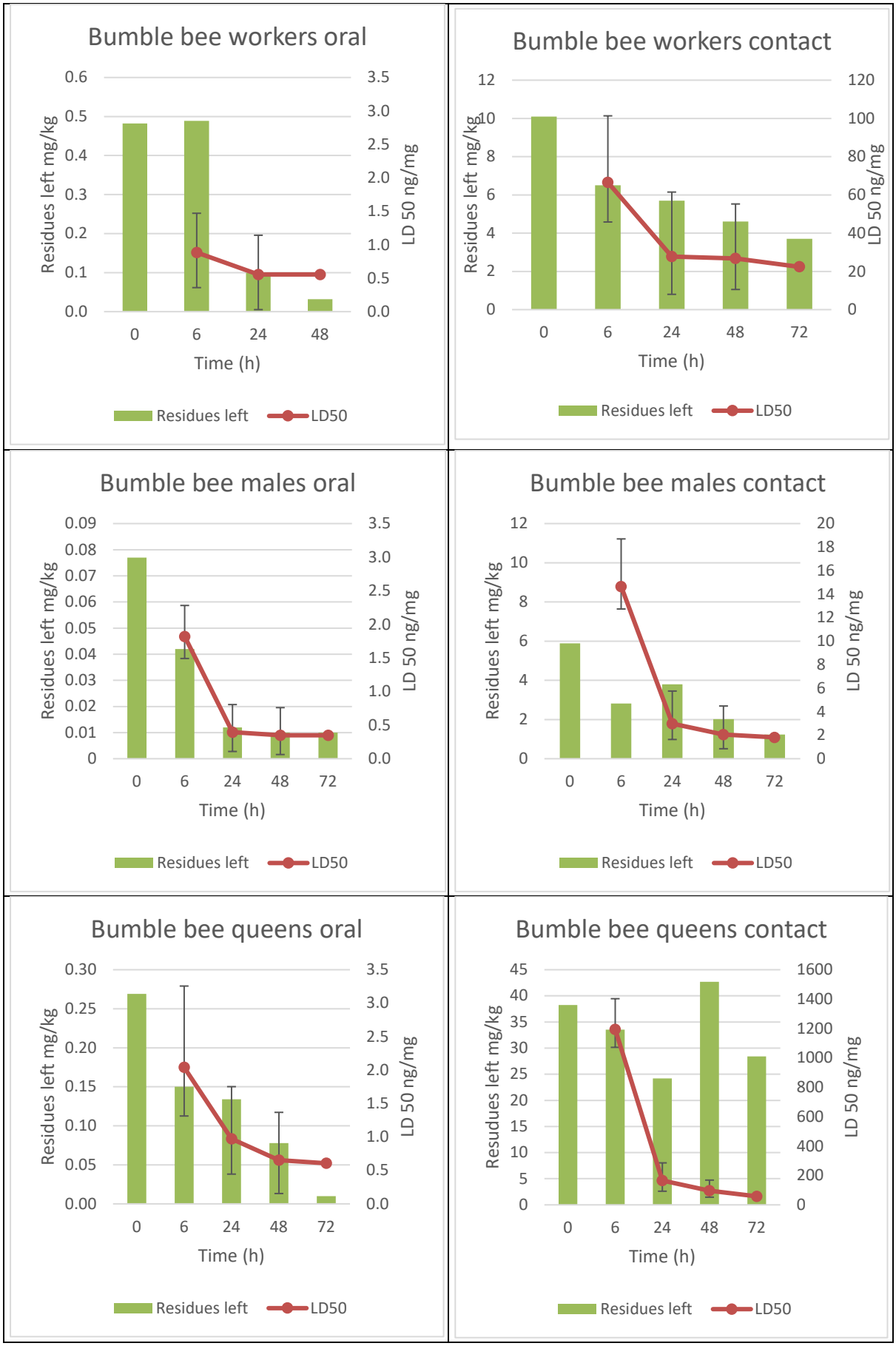


**Figure 4: Concentration of sulfoxaflor residues (mg/kg) and toxicity of sulfoxaflor (LD50s) for different time points in *Bombus terrestris* workers, males and queens after oral (left) and contact exposure (right). Bees were sampled at different time points after exposure**.

**Table 4: The clearance % of sulfoxaflor (dose: NOEL) exposed to oral and contact treatments in bumble bee workers, males and queens within 72, except for oral treatment of workers (48 h)**

| Castes | Clearance % | |
|---|---|---|
| | Oral % | Contact % |
| Worker | 93 | 63 |
| Male | 87 | 79 |
| Queen | 96 | 26 |

### 3.2.2. Amistar/Azoxystrobin

After oral treatment with Amistar the degradation rate was very similar in worker, male and queen bumble bees (Figure 5). The total clearance reached above 80% in all the tested castes (Table 5). After contact treatment, the absolute degradation was lower in all groups (Table 5). The azoxystrobin residues showed a linear decrease until the last time point (72 h) in workers (Figure 5). The initial clearance was faster by 24 h in males, after that time point, the degradation rate slowed down.

The temporal pattern of LD50s after oral treatment in workers and males was very similar to that of sulfoxaflor: despite the fast clearance, there was no significant change in toxicity. In queens, however, despite the slowing down of clearance, the toxicity continues to increase. With contact treatment there was no mortality caused by the used dose of azoxystrobin to any of the castes tested.

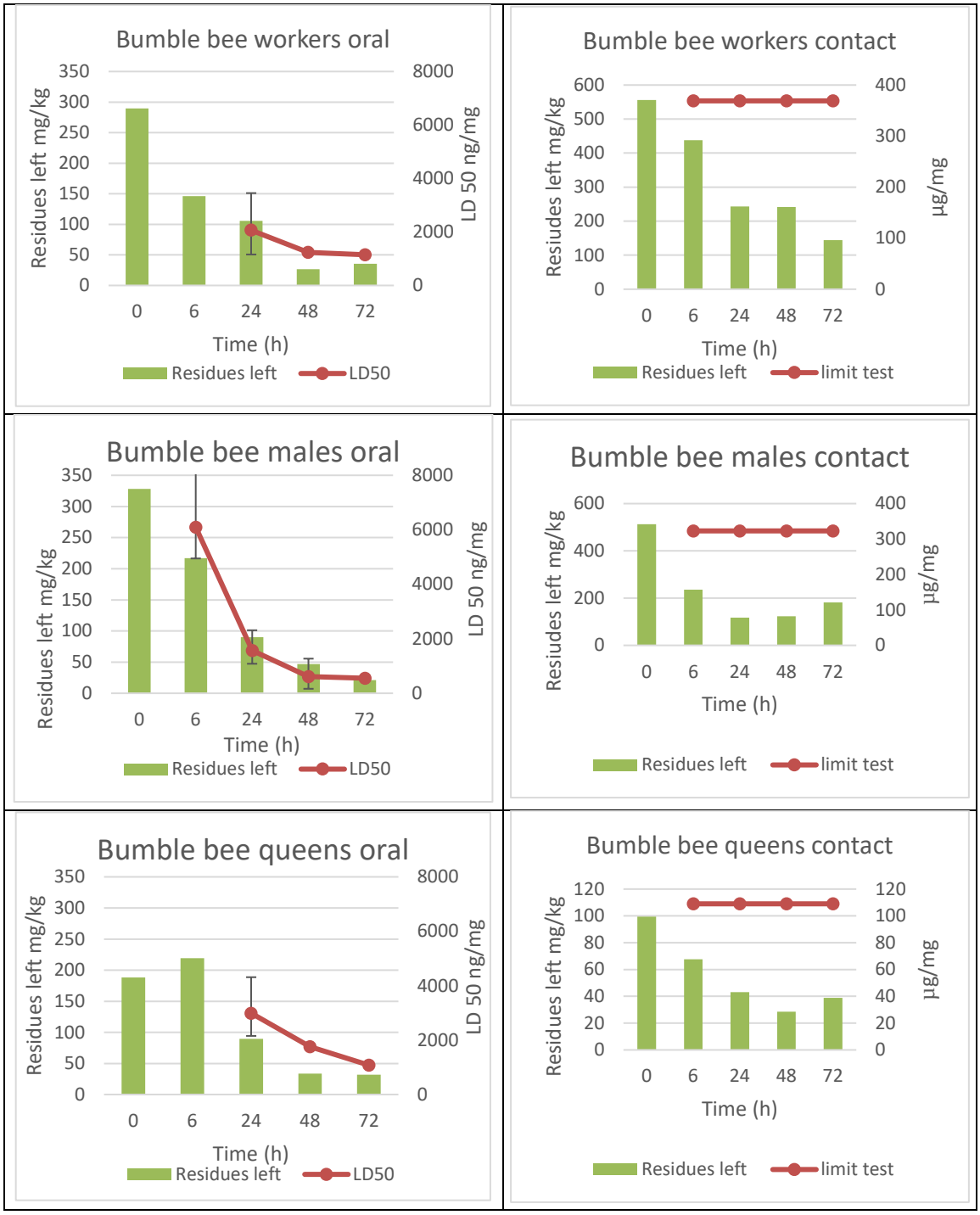


**Figure 5: Concentration of Amistar residues (mg/kg) and toxicity of Amistar (oral) and azoxystrobin (contact) for different time points in *Bombus terrestris* workers, males and queens after oral (left) and contact exposure (right). Bees were sampled at different time points after exposure.**

**Table 5: The clearance % of Amistar/azoxystrobin (dose: NOEL, limit test) exposed to oral and contact treatments in bumble bee workers, males and queens within 72h**

| Castes | Clearance % | |
|---|---|---|
| | Oral % | Contact % |
| Worker | 88 | 74 |
| Male | 94 | 65 |
| Queen | 83 | 61 |

### 3.2.3. Glyphosate

After oral treatment with glyphosate the absolute clearance was variable across groups, being notably low in males (Figure 6, Table 6). In workers by 48 h most of the glyphosate had disappeared. In males the initial increase until 24h was followed by a linear decrease of the residues. In queens, a rapid decline was observed by 6h, but the low level of residues seemed to be persistent for a longer period.

After contact treatment, the absolute degradation was lower in all groups, and particularly so in workers (Table 6). We saw almost linear patterns in all tested bumble bee castes.

Glyphosate poses low toxicity to bumble bees. We did not see mortality in any of the bumble bee castes even though the oral and contact clearance patterns and rates varied.

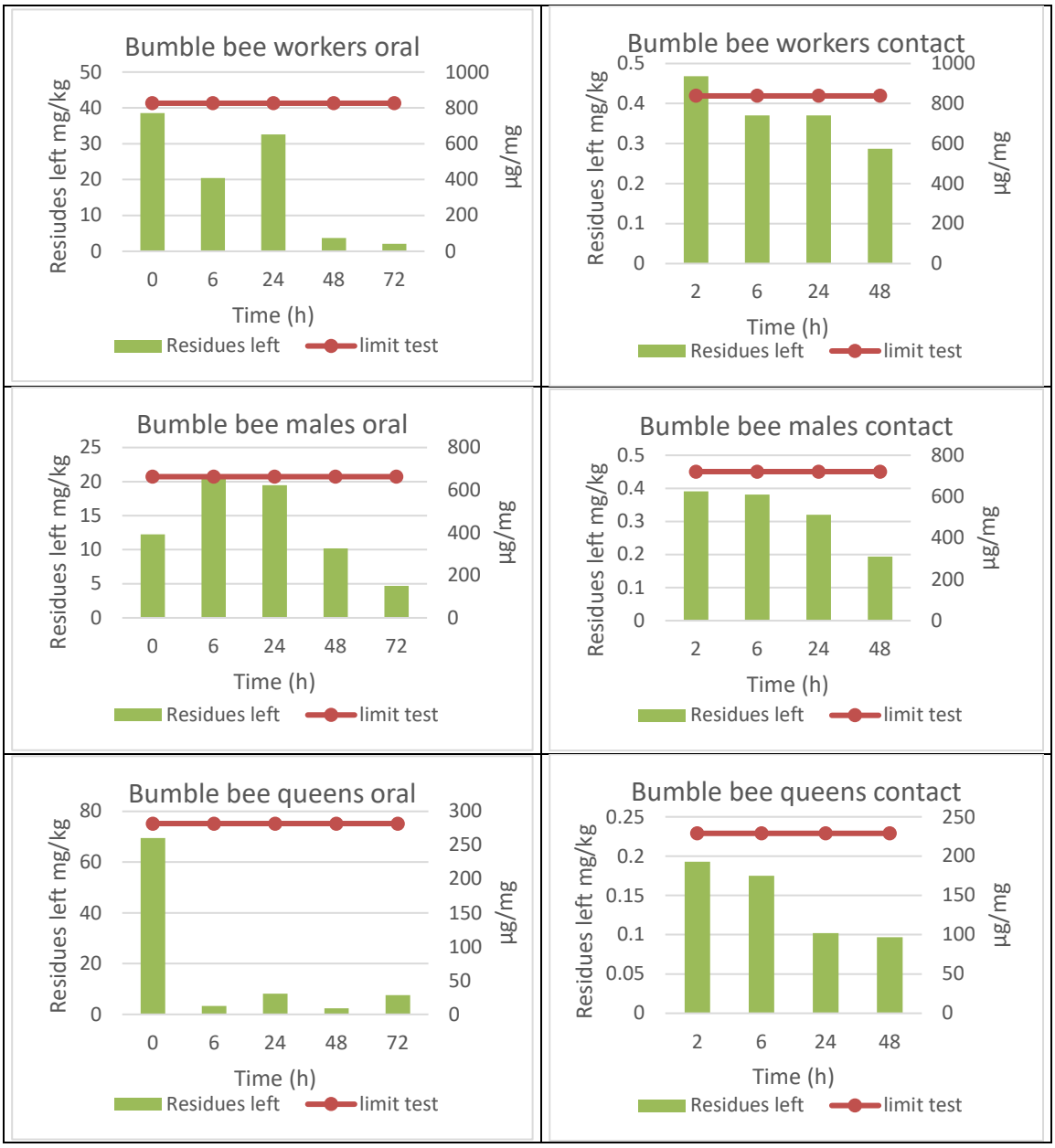


**Figure 6: Concentration of glyphosate residues (mg/kg) and toxicity of (oral) and (contact) for different time points in *Bombus terrestris* workers, males and queens after oral (left) and contact exposure (right). Bees were sampled at different time points after exposure.**

**Table 6: The clearance % of glyphosate (limit test) exposed to oral and contact treatments in bumble bee workers, males and queens within 72h for oral and 48h contact treatment**

| Castes | Clearance % | |
|---|---|---|
| | Oral % | Contact % |
| Worker | 95 | 39 |
| Male | 62 | 50 |
| Queen | 89 | 50 |

## 3.3. Solitary bee *Osmia bicornis*

### 3.3.1. Sulfoxaflor

After oral treatment, there was a rapid initial degradation in females, whereas in males the degradation was relatively linear (Figure 7). Absolute levels of degradation were roughly the same in both sexes (Table 7). In contrast, after topical treatment there was no initial change in residues in females until 6h post-exposure, after which there was a slow decline (Figure 7). Males exhibited an immediate decline after exposure, and then plateaued (Figure 7). Absolute levels of degradation were three times lower in females in comparison to oral exposure, whereas for males the levels remained the same (Table 7).

After oral treatment, although we saw around 70% clearance of sulfoxaflor in both females and males, toxicity increased slowly over time. After contact treatment, the sulfoxaflor appeared to be more toxic from 24h to 48h. Despite the low level of degradation after topical treatment in females, the LD50 pattern resembles that of males, where we saw a rapid degradation.

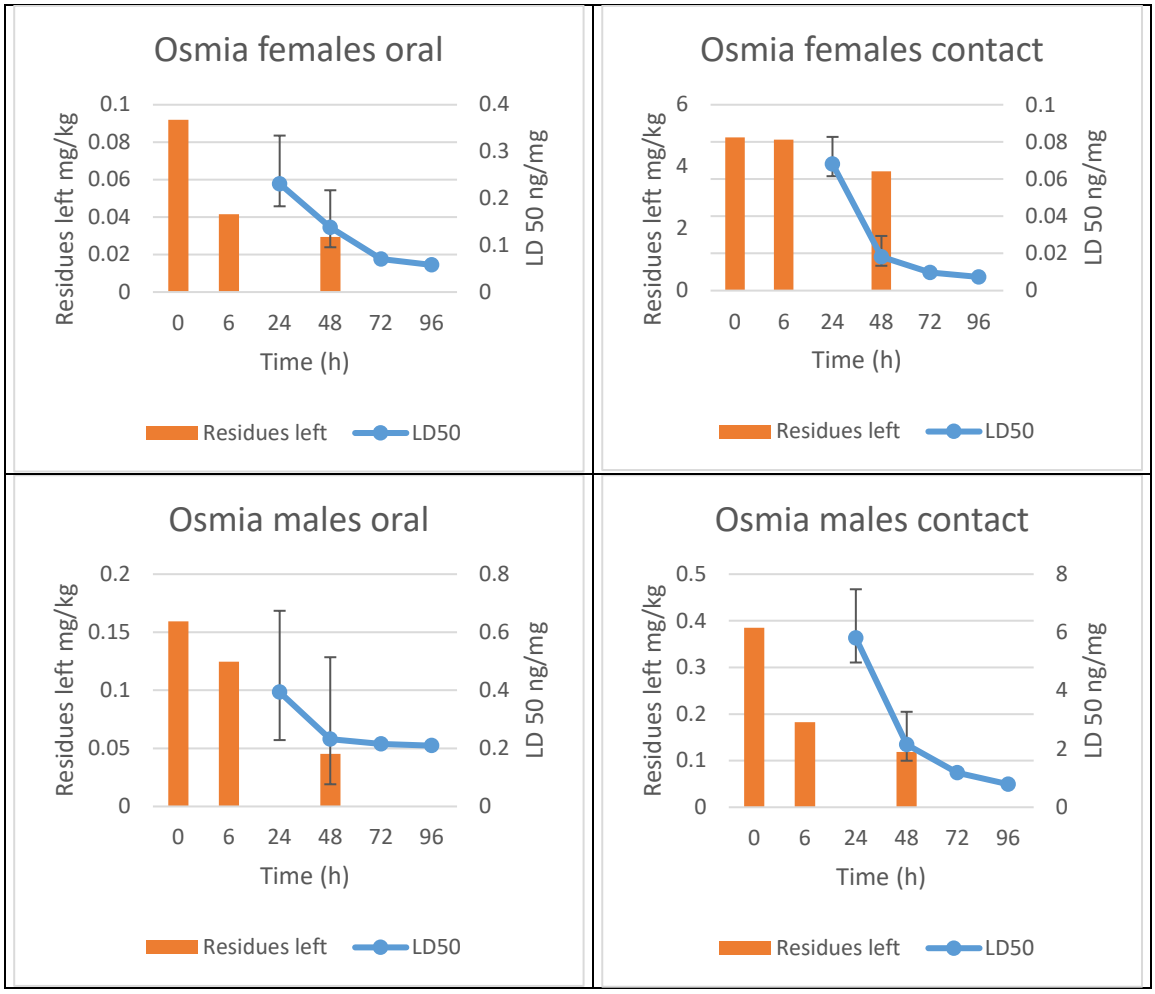


**Figure 7: Residues (ng/bee) in Sulfoxaflor-exposed *Osmia bicornis* females (left) and males (right) sampled at different timepoints**.

**Table 7: The clearance % of sulfoxaflor exposed to oral and contact treatments in *Osmia bicornis* females and males within 48h**

| Sexes | Clearance % | |
|---|---|---|
| | Oral % | Contact % |
| Female | 68 | 23 |
| Male | 72 | 69 |

### 3.3.2. Amistar/Azoxystrobin

After oral treatment, in both females and males there was almost absolute clearance of the azoxystrobin (Figure 8, Table 8). After contact treatment the clearance was not so complete, but similar in both sexes.

The temporal pattern of LD50s were also very similar for both sexes. Despite the rapid degradation, the LD50 pattern varies in time after oral treatment. The LD50 values were more than twice as high after 48h compared to either 24h or 72h. Despite the clearance of the residues from the bodies, the toxicity increases over a longer course of time. The contact treatment with the tested dose of azoxystrobin did not cause any mortality.

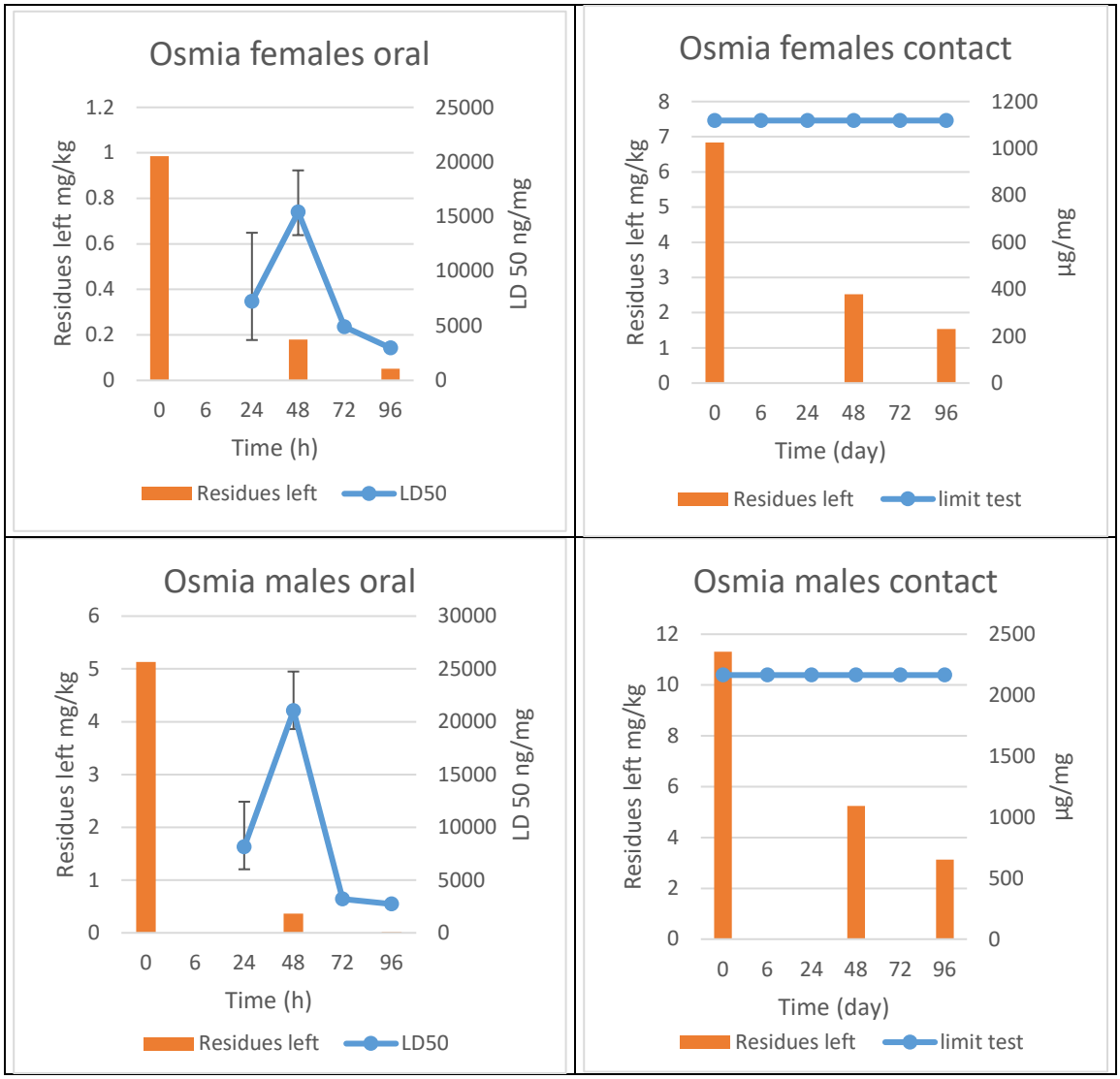


**Figure 8: Residues (ng/bee) in azoxystrobin-exposed *Osmia bicornis* females (left) and males (right) sampled at different timepoints.**

**Table 8: The clearance % of azoxystrobin exposed to oral and contact treatments in *Osmia bicornis* females and males within 96h**

| Sexes | Clearance% | |
|---|---|---|
| | Oral % | Contact % |
| Female | 95 | 78 |
| Male | 99 | 72 |

### 3.3.3. Glyphosate

After oral treatment the clearance of glyphosate was faster in females showing an almost linear pattern during 96h (Figure 9, Table 9), while in males there was almost no clearance within the same time frame. In contact treatment, the clearance in females up to 48h is similar to that after oral exposure. In males however, the clearance was almost complete within 48h with no further decrease over time. The LD values were not calculated because of the low toxicity of glyphosate.

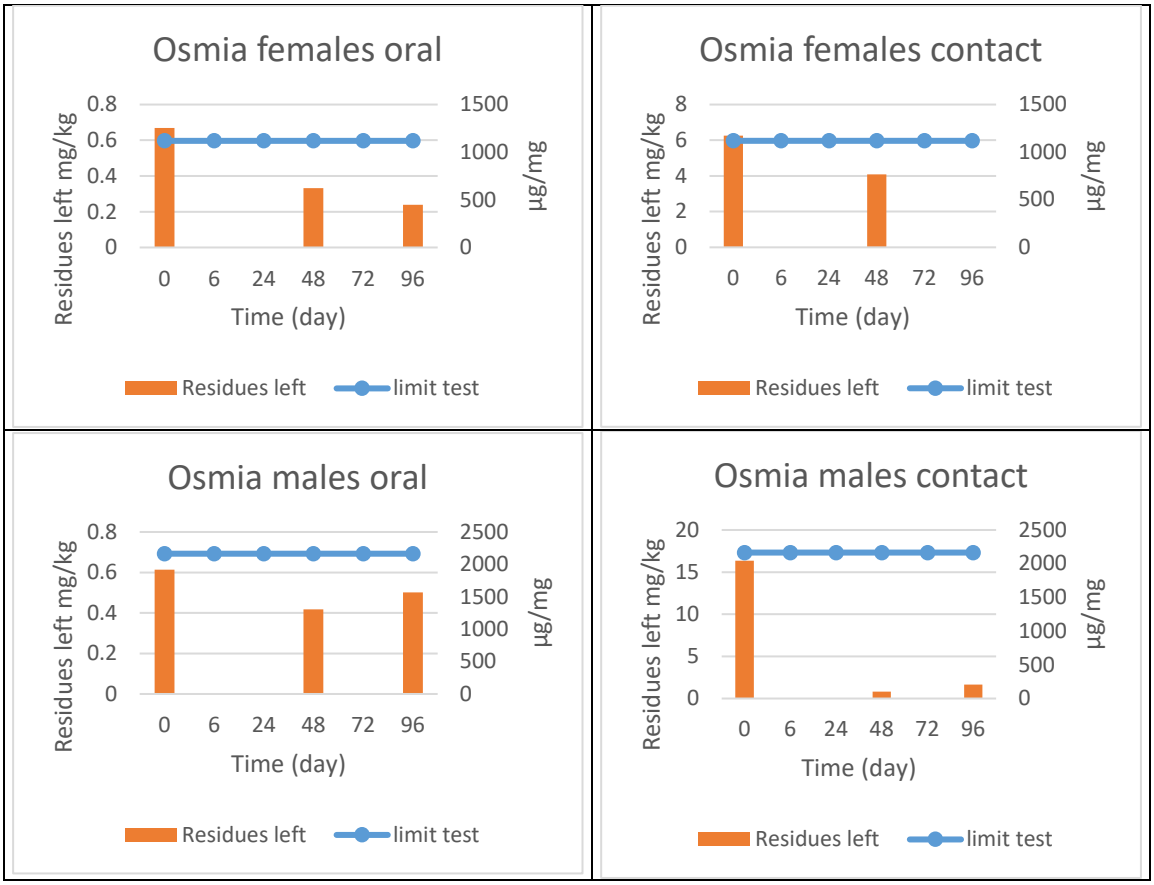


**Figure 9: Residues (ng/bee) in glyphosate-exposed *Osmia bicornis* females (left) and males (right) sampled at different timepoints.**

**Table 9: The clearance % of glyphosate exposed to oral and contact treatments in *Osmia bicornis* females and males within 96h**

| Sexes | Clearance % | |
|---|---|---|
| | Oral % | Contact % |
| Female | 64 | 35 (48h) |
| Male | 18 | 90 |

## 4. Discussion

This study gives good evidence for large variation in physiological as well as behavioural traits not only between honey bees compared to wild bees, but also between different wild bee species. Both the clearance rates and sensitivities of the bees were variable across bee species, sexes/castes and chemical compounds tested. Sulfoxaflor was cleared out of bodies relatively well in bumble bees, but was retained in honey bees and *Osmia* bee females. However, there were caste and treatment specific differences. Azoxystrobin was cleared out relatively well in all bee species, however, honey bee workers were affected by the treatment type. Glyphosate clearance was dependent on the sex/caste and tends to be retained after contact treatment in all bee species tested. Toxicity of a pesticide is dependent on exact dosage, but also the exposure route and time, as well as the speed of detoxification and clearance from a body. The assessment for the hazard that a less toxic pesticide might pose, can be largely dependent on the exposure route as shown here in the case of the fungicide

azoxystrobin. The impacts of pesticide toxicity can increase even after the molecules have cleared out of the body.

The detoxification of xenobiotics involves the metabolization of lipid-soluble substances to water-soluble, excretable metabolites (Berenbaum & Johnson, 2015). This function is primarily reliant on cytochrome P450 monooxygenases (P450) and carboxylesterases (CCE). Transportation of these metabolisation products is also a part of detoxification system. Cytochrome P450 and other detoxification enzymes are present in all insect tissues (Feyereisen, 1999). The acute effects of pesticides in organisms can be reversible or irreversible (Costa et al., 2008) depending on the dosage, physiological and environmental conditions (Muljar et al., 2012).

The insecticide sulfoxaflor is an agonist that binds to acetylcholine receptors (nAChRs) (Watson et al., 2021) leading to full excitation of the synapsis. Sulfoximine (sulfoxaflor belongs to the pesticide class of sulfoximines) chemistry also influences its interactions with monooxygenases (P450s, CYPs) (Watson et al., 2021). The expression patterns of genes coding Cytochrome P450 proteins in honey bees may vary across castes, age groups and body parts (Mao et al., 2015), which might explain differences in the clearance of sulfoxaflor between different castes of bees seen in this study. Also, clearance patterns are probably dependent on metabolic activity, which is highest in insect fat bodies (Arrese & Soulages, 2010; Li et al., 2021), however this might be dependent on exposure routes. In contrast to other castes and sexes, sulfoxaflor residues were retained in *B. terrestris* queens and *O. bicornis* females after contact exposure. The reason for this remains unclear and deserves further study, as it may have significant implications for risk assessment of this chemical.

Azoxystrobin and other strobilurins inhibit mitochondrial respiration by blocking electron transport (Becker et al., 1981; Uçkun & Öz, 2021), which leads to slowing down of any energy-demanding processes. Despite this, we saw high clearance rates in bumble bees only, and not in honey or *Osmia* bees, however the outcome was sex/caste and treatment specific. We can only suppose that these differences may emerge from physiological differences. The variation in clearance rates after contact and oral exposure routes might also be related to the accumulation of the substance in the fat body of the honey bees or the impact of azoxystrobin on bee gut microflora (Lu et al., 2019). We cannot exclude the possibility that the clearance differences in oral and contact treatment came from using Amistar instead of pure a.i. for oral tests. Azoxystrobin is considered to have low toxicity to honey bees (Tamburini et al., 2021) however our results indicate some hazard due to oral exposure. However, the molecule still has potential to affect bees (Christen et al., 2019) indicated by the hormonal disturbance caused by azoxystrobin in adult honey bees, suggesting possible failures from transition of nurse bees to foragers. Such sublethal effects can contribute to the weakening of the whole colony without any notable mortality.

Glyphosate causes low mortality in bees as in other insects, but it changes the microbial communities (Motta et al., 2018) and this might have decreased its degradation in honey bee queens, bumble bees and *Osmia* females, mostly after contact treatment. It remains unclear why, after oral exposure, the glyphosate was not cleared out from the female honey bees, while it was almost lost in drones, and in parallel, there was no similar pattern in either of the wild bee species. The toxicokinetic pathways of glyphosate in insects are clearly understudied and need more scientific attention. The low toxic compound glyphosate is known to cause feeding or sensory failures in honey bees (Herbert et al., 2014). Similarly to azoxystrobin and its formulation, the glyphosate formulations can cause mortality as shown in bumble bees (Straw et al., 2021).

Our results highlight large variation in degradation rates and sensitivity across bumble bees, solitary bees, and honey bees, and across different castes and sexes. Ultimately, these are likely linked to the evolution of different life-histories within and across species (Jackson et al., 2020; Lozier et al., 2021).

There is a clear need for further work on the patterns and processes of detoxification in bees, and its links to lethal and sub-lethal impacts of agrochemicals.

## 5. References


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# 6. Annex 1

## 6.1. Acute oral dose-response relationship (Acute toxicity test)

(BERN: *Verena Strobl, Orlando Yanez, Peter Neumann*)

Newly emerged adult drones, *Apis mellifera mellifera*, were used for this experiment. Drones were orally exposed to the different chemicals, following a modified worker protocol. Briefly, newly emerged drones were first starved for 12h (30 ± 1 °C, 60 ± 10 % relative humidity) before being fed 5 µl feeding solution and then randomly assigned to a treatment. After exposure, drones were kept in hoarding cages (10 drones each) together with 20 workers to maintain drone attendance. After exposure, all cages were fed sugar syrup (50% w/v sucrose) *ad libitum* and syringes with sugar syrup were changed each day to avoid mould growth. We used four cage replicates per treatment. The test conditions were 30 ± 1 °C, 70 ± 10 % relative humidity. Mortality was assessed 4 and 24 hours post-exposure and then every 24 h until termination of the experiment. The $LD_{50}$ was calculated for commercial formulation Amistar (azoxystrobin as active substance, Figure 1) and sulfoxaflor (SFX, Figure 2) as the pure active substance. The assessment of $LD_{50}$ was performed with five increasing doses (from 0.009375 to 0.15 µg/bee in SFX and from 6.25 to 100 µg/bee in Amistar). Two negative controls (one with water and one with acetone) and one reference chemical (dimethoate) were included in the experiment. We used a two parametric logistic function (LL2.2) for the plots and LD50 value calculation.

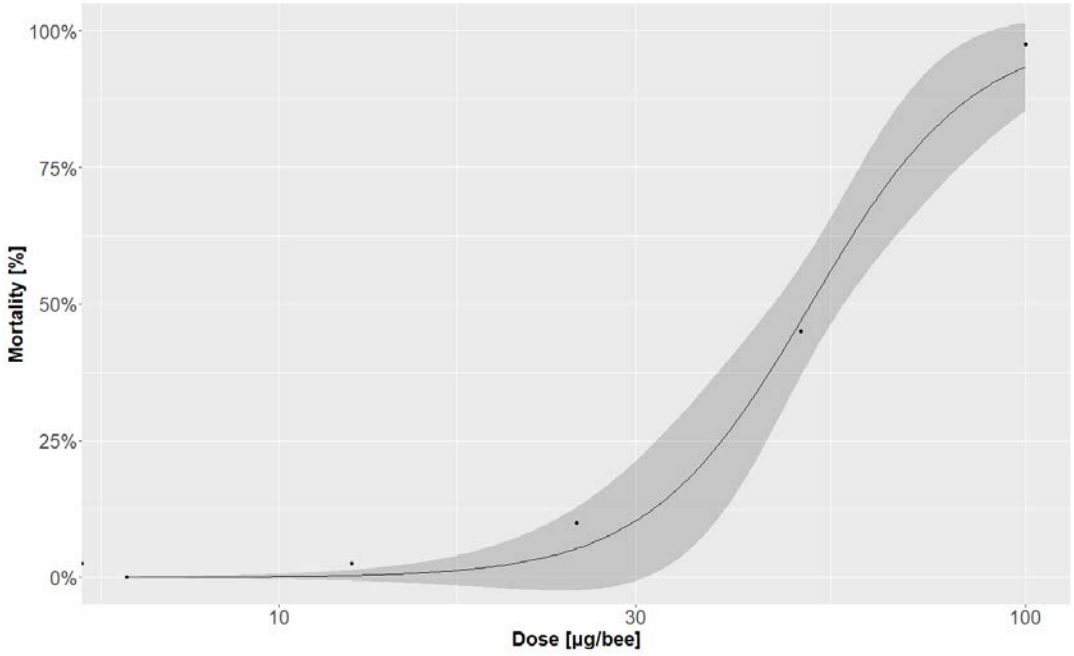


**Figure 1*:* Acute oral toxicity of Amistar (48h) in adult drones of *A. mellifera mellifera*. $LD_{50}$ = 51.6208 µg/bee.**

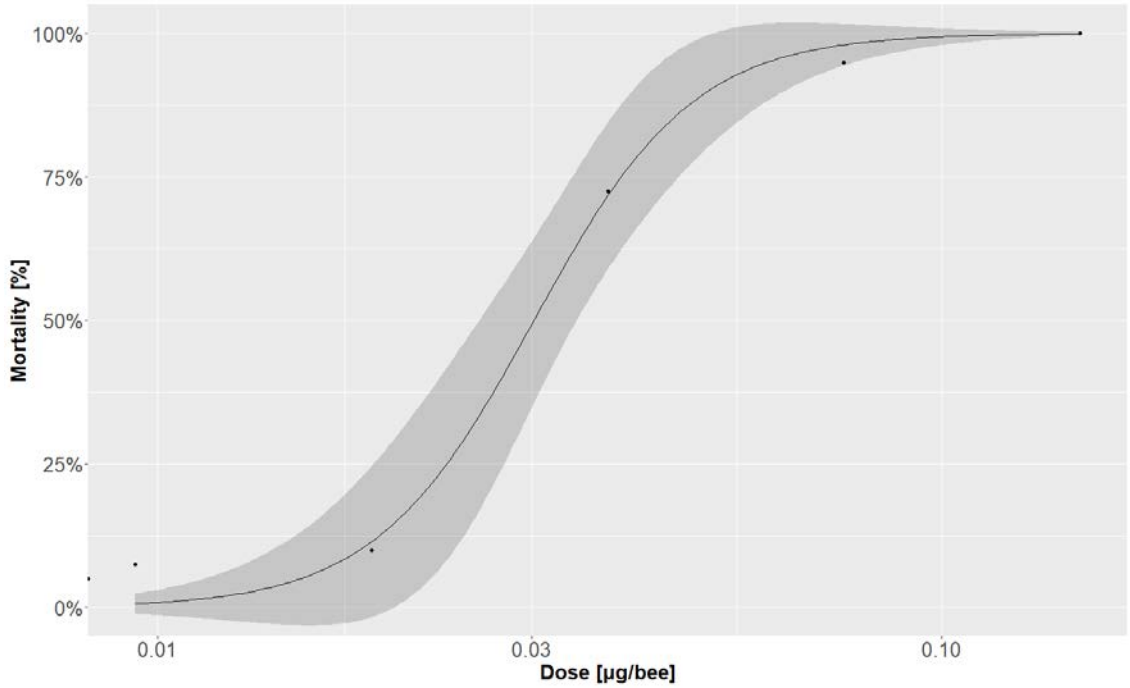


**Figure 1: Acute oral toxicity of sulfoxaflor (48h) in adult drones of *A. mellifera mellifera*. $LD_{50}$ = 0.0303 µg/bee.**

The toxicity of glyphosate was tested with the commercial formulation Roundup Profi (100 µg/bee). Limit tests instead of $LD_{50}$ were used given the low solubility of this substances in acetone and the low presumed toxicity. Roundup Profi (glyphosate) caused 0 % mortality at 48 hrs post-exposure.

Chronic oral toxicity test

Following a modified worker protocol, newly emerged drones (10 each) were placed into a hoarding cage together with 20 newly emerged worker bees for drone attendance (30 ± 1 °C, 60 ± 10 % relative humidity). We used 8 cages per treatment. Cages were instantly exposed for 12 days (so the drones could reach sexual maturity) to the pesticide (active substance: sulfoxaflor (SFX)) via contaminated sugar syrup (50% w/v sucrose, 0.1% acetone) that was fed in a syringe. We exposed bees to a concentration that was considered to be field realistic:

• Sulfoxaflor (0.01 mg/kg)

Sulfoxaflor degrades rapidly (see Deliverable D.3.3). Hence, to ensure the right concentration in the feeding solution, the bees were provided with fresh feeding solutions of the respective treatment daily (always in a new syringe). The survival of a total of 80 drones and 160 workers per experimental group was recorded for 12 days (every 24h). There was no evidence of treatment effect on mortality on drones (Figure 3) nor on the attendance workers (Figure 4). To asses mortality, we r~~a~~un a "survfit" model (R packages "survival" and "survminer"). After running and plotting the model, we used chi squared test to look for significant differences.

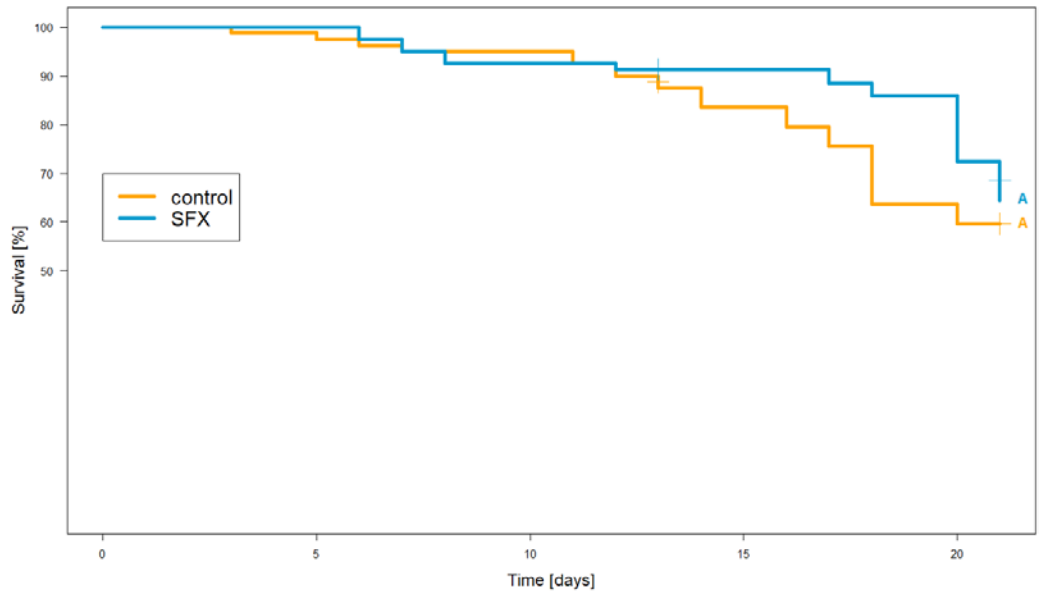


**Figure 2: Effects of pesticides on the survival of adult drones caged with workers, *Apis mellifera mellifera*. Data show survival probability over 21 days (n=8 cages of 10 bees per treatment) in controls and sulfoxaflor (SFX) treatments. Same letters indicate no significant differences between treatments (χ2 = 0.8, p = 0.4).**

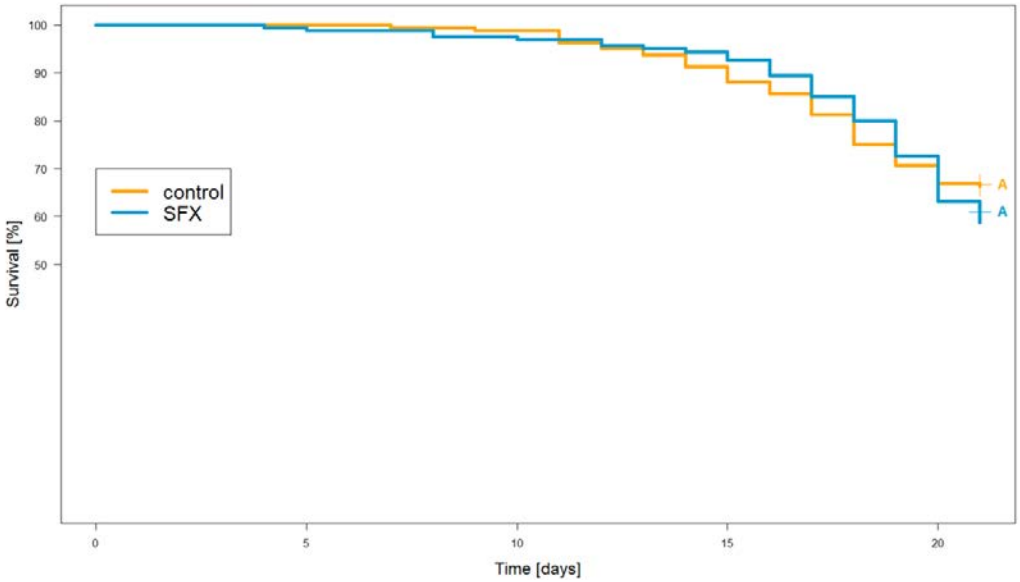


**Figure 4: Effects of pesticides on the survival of workers caged with adult drones, *Apis mellifera mellifera*. Data show survival probability over 21 days (n=8 cages of 20 bees per treatment) in controls and sulfoxaflor (SFX) treatments. Same letters indicate no significant differences between treatments (χ2 = 0.9, p = 0.3).**

After the 12 day exposure period, a subsample of surviving drones from each treatment was randomly taken, and used for the sperm assessment. After carefully removing the drones from the cages using forceps, we dissected them following Straub et al. (2016). Briefly, after pinning the drones to a wax plate, they were dissected alive to prevent sperm from migrating into the penis bulb. The entire reproductive tract including the testes, mucus glands and seminal vesicles was removed from each drone following Carreck et al. (2013) and then gently crushed in a Kiev$^{+}$ buffer. Using light microscopy, sperm quantity was assessed. Sperm viability (percentage of alive sperm) was assessed using

fluorescence microscopy. Sperm quantity did not significantly vary among the two treatments (Figure 5). However, drones from the sulfoxaflor cages showed a significantly lower sperm viability than control groups (Figure 6). To analyse whether there are significant differences between the treatments, we run a one-way ANOVA. To figure out how significantly the groups differ, we used a Tukey multiple comparisons of means 95% family-wise confidence level.

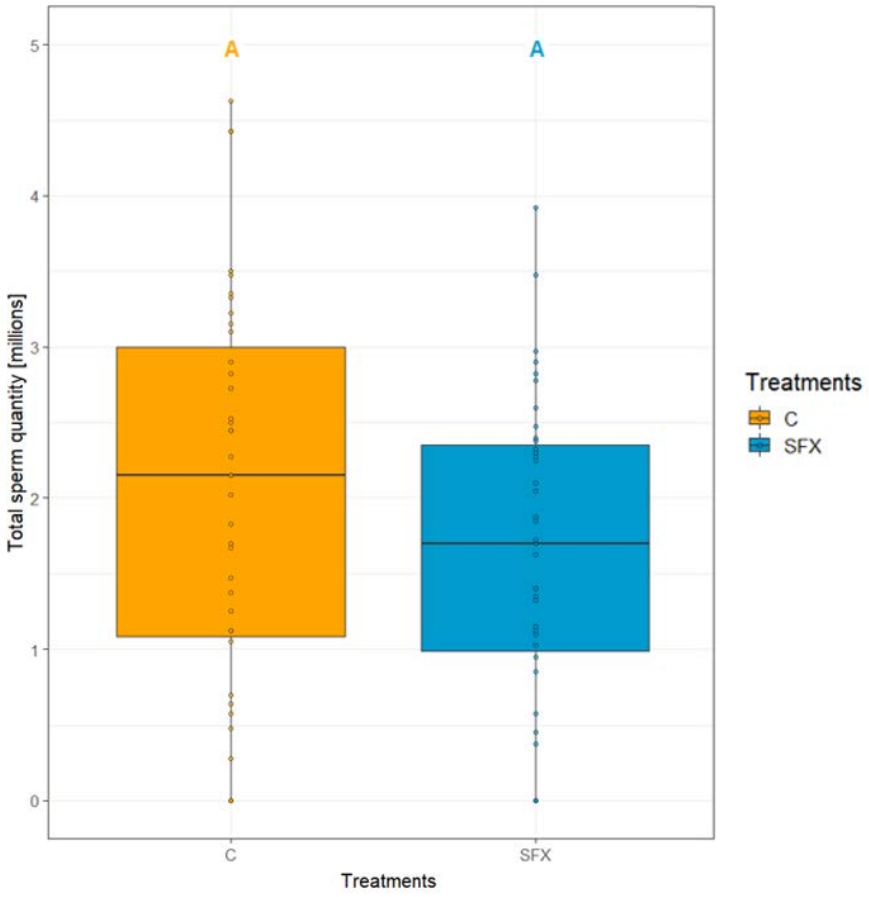


**Figure 5**: **Sperm quantity. Data show the sperm quantity of adult honey bee drones (*Apis mellifera mellifera*) in controls (C) and sulfoxaflor (SFX) treatments. Same letters indicate no significant differences between treatments (F(2, 144) = -0.384, p = 0.468, 95% C.I. = -1.037, 0.269])**

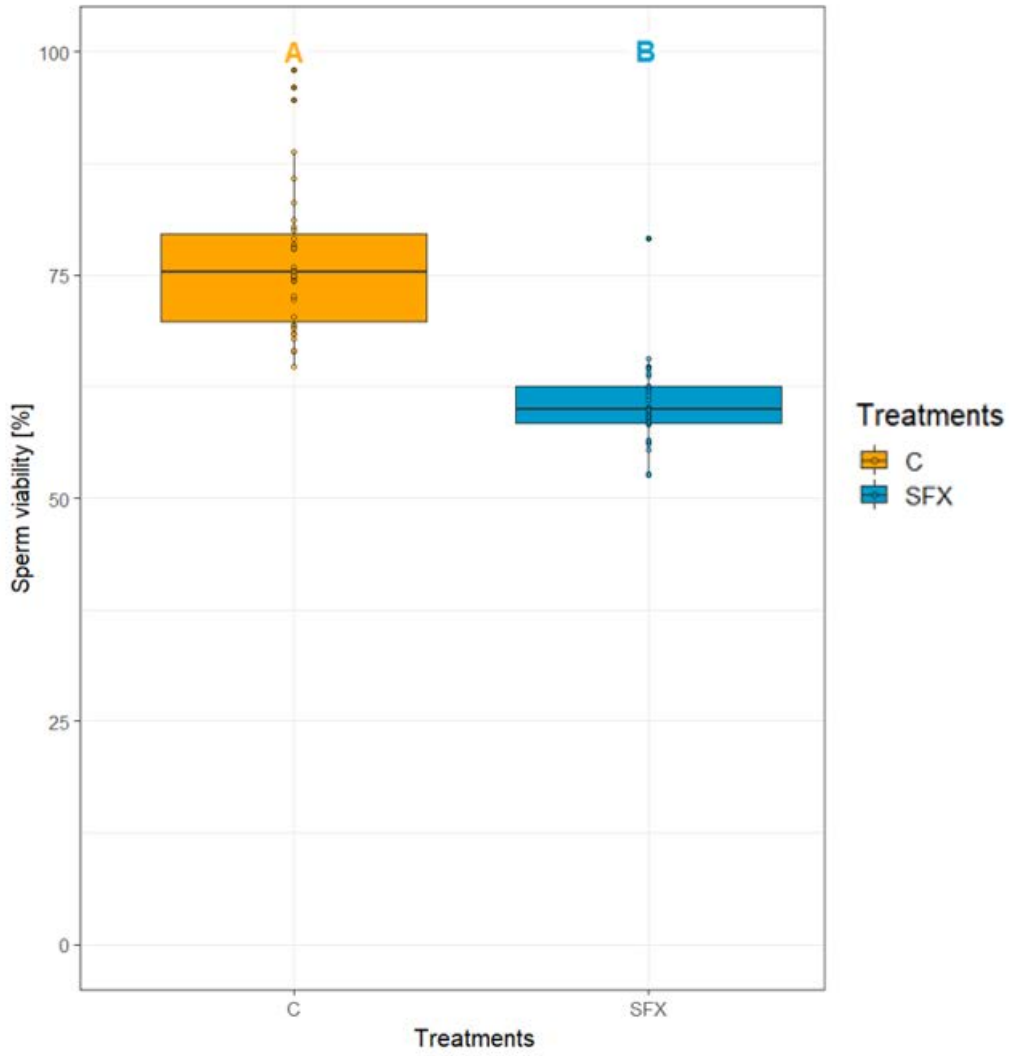


**Figure 3. Sperm viability. Data show the sperm viability of adult honey bee drones (*A. mellifera mellifera*) in controls (C) and sulfoxaflor (SFX) treatments. Different letters indicate significant differences between treatments (F(2,131) = -16.025, p < 0.0001, 95% C.I. = [-20.382, -11.669])**

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# 7. Annex 2

## 7.1. Acute oral toxicity of sulfoxaflor on honey bee queens

(BERN: *Verena Strobl, Orlando Yanez, Peter Neumann*)

Oral dose-response in *Apis mellifera* queens

The acute oral toxicity of sulfoxaflor on adult honey bee queens, *Apis mellifera mellifera*, was tested by applying a dose-response design using improved protocols for testing agrochemicals in bees (Deliverable D3.2). We calculated LD values for different time points. We performed queen rearing with three *A. mellifera mellifera* colonies to obtain purebred queens for the experiment. The queen rearing and grafting procedure was done based on Büchler *et al*. (2013) with some minor adaptations. The queen rearing consists of three major steps. First, larvae are transferred from their original cell into an artificial cell in a process called grafting. The grafted larvae are then placed in special frames into nursing colonies, which rear them into becoming queens. After 5 days the cells are closed and can be taken out of the nursing colonies. The cells on one frame are isolated using emergence cages and placed in the incubator at 34.5°C and 80% humidity until emergence of the queens when they are 10-12 days old.

Freshly emerged queens were starved for 2 hours before exposure. The queens (N=70) were randomly assigned to one of six treatment groups; five ascending concentrations of sulfoxaflor and one acetone control group. The different concentrations for each treatment are listed in Table 2.1. After the starvation period, each queen was orally exposed according to treatment group. The exposure was performed by feeding the queens with 2 µl of 1:1 sugar water containing different concentrations of Sulfoxaflor (S1-S5) or acetone control. After exposure the queens are housed in nicot queen cages (Nicotplast, FR) together with 8 freshly emerged workers. The cages were stocked with a 1:3 spring honey – powder sugar mixture. After exposure, the mortality of queens and workers were recorded. Based on queen mortality LD50 values were calculated for different time points (Table 2.2). The calculations for the LD50 were done using a two parametric logistic function (R, version 4.1.2) using a two parameter logistic function with mortality as the dependent variable and concentration as the independent variable.

**Table 1: Sulfoxaflor concentrations for each treatment group [µg/bee]**

| Treatment | Sulfoxaflor concentration |
|---|---|
| C | 0 µg/bee |
| S5 | 0.15 µg/bee |
| S4 | 0.075 µg/bee |
| S3 | 0.0375 µg/bee |
| S2 | 0.01875 µg/bee |
| S1 | 0.00938 µg/bee |

**Table 2: Acute oral LD50 (95% CI) of sulfoxaflor for adult honey bee queens, *Apis mellifera mellifera*.**

| Assessment time [h] | LD50 value [µg/bee] |
|---|---|
| 4 | 0.143 (0.067-0.305) |
| 24 | 0.132 (0.063-0.274) |
| 48 | 0.128 (0.063-0.262) |
| 72 | 0.128 (0.063-0.262) |
| 96 | 0.127 (0.062-0.258) |

Reference

Büchler, R., Andonov, S., Bienefeld, K., Costa, C., Hatjina, F., Kezic, N., …, (2013), Standard methods for rearing and selection of *Apis mellifera* queens. *Journal of Apicultural Research*, *52*(1):1-30. https://doi.org/10.3896/IBRA.1.52.1.07.