Manuscript of toxicokinetics of three agrochemicals in three model bee species

Deliverable D3.1

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Cecilia Costa[1], Gennaro Di Prisco[2], Cedric Alaux[3], Piero Onorati[4], Margret Jürison [5], Reet Karise [5], Marika Mänd[5]; Sara Hellström[6]; Piotr Medrzycki[2]

[1] CREA – Agriculture and Environment Research Centre, Italy; [2] IPSP-CNR; [4] SLU - Swedish University of Agricultural Sciences [5] Estonian University of Life Sciences; [6] Martin Luther University

PoshBee

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

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Table of contents

Summary

As part of the activities foreseen by the POSHBEE project, we performed experiments to verify the toxicokinetics of three pesticides (the insecticide sulfoxaflor, the fungicide azoxystrobin and the herbicide glyphosate) in the bodies of social bees (*Apis mellifera* and *Bombus terrestris*) and solitary bees (*Osmia bicornis*). For each species all castes and sexes were studied. Based on the results from dose-response assays, sublethal doses were used to treat individuals, which were exposed to the chosen pesticides orally and by contact. Bees were then sampled at multiple time points post-exposure, to capture the breakdown of the active ingredients in the bodies of the organisms. Results of the chemical analyses on bee samples were used for the evaluation of the dynamics of the oral and contact acute exposure in the three species.

Overall, the active ingredients sulfoxaflor, azoxystrobin and glyphosate degrade in all species, sex or caste. Nevertheless, there were some exceptions: in honey bee workers, glyphosate administered topically and azoxystrobin administered via the oral route seemed to remain stable in the bees' bodies even 10 days after exposure (11% and 13% of degradation rate, respectively). We also observed a low degradation of sulfoxaflor following topical exposure in bumble bee queens (26% of degradation rate) and in *Osmia* bee females (22% of degradation rate). In bumble bees exposed topically to glyphosate degradation was lower than 50% for all sexes and castes. These results deserve further attention by researchers to understand the destiny of these molecules in the bee body and their effects therein.

1. Introduction

Globally, pollinators are responsible for pollinating more than 1,200 crops and about 75% of the leading world food crops depend on pollinators (Klein et al., 2007). Pollinators provide pollination services to over 180,000 different plant species, contributing to environmental and societal benefits, which include producing an important part of our food supply, providing food and cover for wildlife, preventing soil erosion, producing the oxygen we breathe, and absorbing C02, thus counteracting global climate change (Potts et al. 2016).

Bees (superfamily Apoidea) are the most effective group of pollinators, with approximately 20,000 species worldwide and 2,000 species in Europe (IPBES, 2016). The European honeybee (*Apis mellifera* L.) is the most widely managed pollinator species contributing to crop pollination (Aizen and Harder, 2009), but both managed and wild bee species contribute significantly to crop pollination globally (Garibaldi et al. 2013).

In recent years, honey bee populations have been subject to decline and beekeepers have suffered unprecedented losses (Brodschneider et al, 2016). This decline has been linked to several factors, including parasites and viral infections, monoculture farming which prevents bees from having a varied diet, and the use of pesticides which can have toxic effects on bees. Each year many honey bee colonies are damaged or destroyed by pesticides, primarily insecticides. Negative effects likely affect all pollinators.

Before being placed on the market, pesticides are evaluated for their impact on human health and the environment. In ecotoxicological studies honey bees for a long time were the only considered species representing the superfamily Apoidea, and only the acute toxicity of substances was considered. Recently the pesticide risk assessment scheme in the EU has been revised to include other species (bumble bees and solitary bees), different development stages (adults and larvae) and to consider sublethal and chronic effects. In ecotoxicological tests a defined exposure is always

assured. However, substances may also differ in terms of how rapidly they may be degraded in the bee's body, and insights into these mechanisms may provide useful input for further reviewing the test protocols for evaluating the impact of a certain chemical.

Within the POSHBEE project, DL50s of selected active ingredients sulfoxaflor (insecticide), azoxystrobin (fungicide) and glyphosate (herbicide) were determined (Poshbee deliverable D3.2 Alaux et al, May 2022).

These substances were chosen because:

- the insecticide sulfoxaflor seemed to be replacing the banned neonicotinoids across the EU and beyond, being a systemic insecticide with a similar mode of action as neonicotinoids (nicotinic acetylcholine receptor (nAChR) competitive modulators), and thus may have similar negative effects on pollinators;
- the fungicide azoxystrobin is one of the most widely used fungicides. Its mode of action is inhibition of the respiration system, which can also affect insects, and its usage coincides with sulfoxaflor and hence might threaten bees with mixed exposure;
- the herbicide glyphosate is the most widespread and used pesticide in the world, while the impact on non-target organisms is still unclear and controversial.

In this report we summarize the methods and results of experiments performed to determine the dynamics of the active ingredients' metabolization within individual bees, following their exposure to sub-lethal doses, which were chosen using data obtained from determination of LD50s of the model active substances sulfoxaflor and azoxystrobin (D 3.2 Alaux et al,). The three model bee species: *Apis mellifera*, *Bombus terrestris,* and *Osmia bicornis* were used, including in some cases different castes and sexes. In bumble bees, tests were performed on workers, males, and queens, exposed orally and by contact. In honey bees, tests were performed on workers and queens exposed orally and by contact, and drones exposed by contact. In *O. bicornis*, both sexes were exposed orally and by contact. The final sampling points reported in this deliverable differ for the different substances and species, due to the different mortality trends and different foreseen breakdown dynamics observed during the dose-response experiments.

2. Toxicokinetic assessment of oral acute exposure

Bees were orally exposed to sublethal doses of the chosen agrochemicals and subsequently groups of bees were sampled at different time points following the exposure.

Samples weighing at least 2 g were used for chemical analyses. For sulfoxaflor and azoxystrobin the substrates were prepared with a simplified QuEChERS method, which consists of an extraction and a purification stage: in the extraction stage, $MgSO_4$ salts together with a solution of water and acetonitrile were added. The samples were centrifuged and the supernatant collected and purified with a PSA resin, before further centrifugation, concentration and a specific solvent added for the LC-MS/MS analysis. Quantification of active ingredients was performed by means of a calibration curve with the standards in solvent, which was conducted at each analytical cycle. For both azoxystrobin and sulfoxaflor LOD was 2 µg/kg and LOQ 10 µg/kg. External calibration in solvent was from 2 µg/kg to 400 µg/kg.

For glyphosate analyses bee samples (2 g), were extracted in aqueous media and analysed by UPLC-MS/MS after precolumn derivatization reaction with 9-fluorenylmethoxycarbonyl chloride (9-FMOC-Cl), with glyphosate-2-13C as Internal Standard. The derivatization reaction was conducted in basic borate buffer at pH 10.5. After derivatization the mixtures were filtered using 25mm PTFE filter (0,45µm pore size) directly in 1.5 ml polypropylene LC vials. The LOQ and LOD for these bees matrix are: LOQ 10 µg/kg, LOQ 3 µg/kg. The quantification was performed at each batch of analysis

derivatization, with a series of calibration standard solution in the concentration range of 5-1000 µg/kg. The samples which exceeded the glyphosate concentration of 1000 µg/kg (higher than the linearity response of glyphosate) were diluted with water prior to the derivatization step and re-derivatized.

2.1. Honey bees

The comprehensive methods employed in the experiment are described in PoshBee deliverable D3.2 by Medrzycki et al., 2021.

2.1.1. Honey bee workers

Apis mellifera ligustica, one of the most widely commercially used honey bee subspecies in Europe and worldwide (Meixner et al., 2010) was used for the experiment. As described in more detail in Medrzycki et al., 2021, newly emerged worker bees were incubated at 33 °C for 3-4 days, while being fed with sucrose solution *ad libitum*. Bees were then randomly allocated to the test cages (30 bees per cage) after being anaesthetised by CO2:air mixture for a short time interval. One cage per time point per replicate was set up, and three replicates were performed. For oral exposure, each cage received a feeder containing a total of 300 microlitres of test feeding solution (=10 µL per bee), for a duration of 4 hours (maximum).

Bees were then fed *ad libitum* with sucrose solution, apart from the sampling points, where the bees were starved for 2 hours before freezing the whole test cage at -18°C. Bee mortality in each cage was assessed and recorded after the exposure, and each time a sample was collected.

Sampling points differed according to the tested substance due to the different mortality trends and different foreseen breakdown dynamics observed during the dose-response experiments.

Levels of residues found in the bee bodies at the beginning of the experiment (after exposure) and at the end are reported. The final time point was 72 hours for sulfoxaflor, 10 days for azoxystrobin and for glyphosate.

Sulfoxaflor was tested in pure (active substance) form - no commercial formulation was used. The test syrup was prepared by adding a 50mg/L sulfoxaflor solution in acetone to the sucrose solution (50%w/v), at a rate of 1%. Thus 10µL (dose per one bee) of this diet contained 5ng of the chemical. Azoxystrobin was tested as commercial formulation Amistar which contains 250g of active ingredient per litre of formulation. The test syrup was prepared by adding Amistar (diluted 1/100 in water) to the sucrose solution (50%w/v), at a rate of 40mL/L. Thus 10µL (dose per one bee) of this diet contained 1µg of the chemical. Glyphosate was tested as commercial formulation Roundup Platinum which contains 480g of active ingredient per litre of formulation. The test syrup was prepared by adding Roundup Platinum (diluted 1/100 in water) to the sucrose solution (50%w/v), at a rate of 20.83mL/L. Thus 10µL (dose per one bee) of this diet contained 1µg of the chemical.

Table 1: Degradation of the active substances in worker honey bees, exposed orally.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor (pure)	0.043	0.023	46%
Azoxystrobin (Amistar)	3.472	3.016	13%
Glyphosate (Roundup Platinum)	0.306	0.148	52%

Table 1 reports the concentration of the active substance found in the samples of honey bee bodies immediately after the 4 hour exposure period (initial level) and then after 72 hours for sulfoxaflor, and after 10 days for azoxystrobin and for glyphosate (final levels). Different final timepoints were chosen due to the different toxicity of the three substances highlighted in the dose-response studies (see PoshBee deliverable D3.3 Alaux et al., 2022). While sulfoxaflor and glyphosate seemed to degrade at high rates (especially sulfoxaflor, with residue levels in bee bodies halved in the final timepoints) azoxystrobin seemed to remain stable in the bee body. This could be due to accumulation in the fat bodies, although specific studies with separation of the different bee organs should be performed to confirm this.

2.1.2. Honey bee queens

Honey bee queens were tested using the same methods as for honey bee workers. Table 2 reports levels of the a.i. immediately after the end of the oral exposure and 24 hours later.

Table 2: Degradation of the active substances in queen honey bees, exposed orally.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor (pure)	0.026	0.011	58%
Azoxystrobin (Amistar)	0.267	0.104	61%
Glyphosate (Roundup Platinum)	3.269	0.776	76%

Degradation was higher in queens than in workers for all a.i., and the low degradation of azoxystrobin found in workers was not noticed in queens, highlighting the physiological differences between the castes.

2.1.3. Honey bee drones

Honey bee drones of the subspecies *A. m. carnica* were tested using the same methods as for honey bee workers. However for honey bee drones there was a high mortality in the cages and it was not possible to collect sufficient individuals for the chemical analyses.

2.2. Bumble bees (*Bombus terrestris*)

The toxicological endpoints and metabolization rate of agrochemicals after oral exposure on workers, males, and queens of *Bombus terrestris* were determined. The comprehensive methods employed in the experiment are described in PoshBee deliverable D3.2 by Medrzycki et al., 2021.
Bumble bees were randomly selected from colonies and evenly distributed by weight (avoiding extremely small and large sized workers). Bees were then individually allocated to Nicot® cages and adapted to the test conditions (25 ± 1 °C, ~60% relative humidity and darkness) for 24 h with *ad libitum* access to untreated 50 % weight per volume (w/v) aqueous sucrose solution. Prior to the test, bees were starved for about 2-6h and dosed individually with test feeding solution (40 µL per bee) for a duration of 4 hours (maximum). Bees were then fed *ad libitum* with sucrose solution, apart from the sampling points, and then frozen at -18°C.

For all substances the reported initial level is the amount of residues in samples collected immediately after exposure, while the final level is residues in samples collected 72 hours after exposure. Levels of residues found in the bumble bee samples at the beginning and at the end of the experiment are reported in Tables 2, 3 and 4.

Sulfoxaflor and glyphosate were tested in pure (active substance) form, while azoxystrobin was tested as the commercial formulation Amistar which contains 250g of active ingredient per litre of formulation.

2.2.1. Bumble bee workers

Each worker bumble bee was treated with 0.08 µg/bee of sulfoxaflor, 80 µg/bee of azoxystrobin, or 200 µg/bee of glyphosate; test substances were added to 40 µL 50% w/v sucrose solution.

Table 3: Degradation of the active substance in worker bumble bees, exposed orally.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	0.482	0.024	95%
Azoxystrobin (Amistar)	289.490	35.495	88%
Glyphosate	38.577	2.043	95%

Table 3 reports the concentration of the active substance found in the samples of bumble bee workers' bodies immediately after exposure period (initial level) and then after 72 hours (final level). Degradation of all the tested substances in bumble bee workers was high, compared to results in honey bee workers.

2.2.2. Bumble bee queens

Each queen bumble bee was treated with 0.18 µg/bee of sulfoxaflor, 350 µg/bee of azoxystrobin, or 200 µg/bee of glyphosate; test substances were added to 40 µL 50% w/v sucrose solution.

Table 4: Degradation of the active substance in queen bumble bees, exposed orally.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	0.269	0.010	96%
Azoxystrobin (Amistar)	188.096	31.752	83%
Glyphosate	69.471	7.560	89%

Table 4 reports the concentration of the active substance found in the samples of queen bumble bee bodies immediately after exposure period (initial level) and then after 72 hours (final level). Degradation of all the tested substances in bumble bee queens was higher than in honey bee workers and comparable to bumble bee workers.

2.2.3. Bumble bee males

Each male bumble bee was treated with 0.02 µg/bee of sulfoxaflor, 80 µg/bee of azoxystrobin, or 200 µg/bee of glyphosate; test substances were added to 40 µL 50% w/v sucrose solution.

Table 5: Degradation of the active substance in male bumble bees, exposed orally.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	0.077	<0.010	87%
Azoxystrobin (Amistar)	328.326	21.328	94%
Glyphosate	12.260	4.699	62%

Table 5 reports the concentration of the active substance found in the samples of male bumble bee bodies immediately after the exposure period (initial level) and then after 72 hours (final level). Degradation of all the tested substances in bumble bee males was higher than in honey bee workers but slightly lower than in bumble bee workers.

2.3. Solitary bees (*Osmia bicornis*)

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

The methods used for exposure and husbandry of the bees are described in detail in Medrzycki, P. et al. (2021). 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.

Males and females were tested separately. The initial time point was at the end of the exposure phase (3 hours) and the final time point was 48 hours from exposure for sulfoxaflor and 14 days for azoxystrobin and glyphosate exposure. The residues per bee at the first and final sampling points are presented in Tables 6 and 7. The degradation rate signifies the residues present in the initial sample divided by that present at the final sampling point.

Table 6: Degradation of the active substances in female *O. bicornis*, exposed orally.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	0.082	0.029	67%
Azoxystrobin	0.986	0.006	>99%
Glyphosate (Roundup ProActive)	0.669	0.189	71%

Table 7: Degradation of the active substances in male *O. bicornis*, exposed orally.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	0.159	0.0454	71%
Azoxystrobin	5.129	0.009	>99%
Glyphosate (Roundup ProActive)	0.614	0.362	41%

As shown in Tables 6 and 7 degradation of azoxystrobin after 14 days was almost complete in both females and males (>99%). Levels of sulfoxaflor were more than halved in 48 hours (67-71%). The degradation of glyphosate was considerably slower, with 71-41% reduction after 14 days.

3. Toxicokinetic assessment of contact acute exposure

Bees were exposed by contact to sublethal doses of the chosen agrochemicals and subsequently groups of bees were sampled at different time points following the exposure. The analytical methods are the same as described for the samples from the oral exposure.

3.1. Honey bees

The comprehensive methods employed in the experiment are described in PoshBee deliverable D3.2 by Medrzycki et al., 2021.

3.1.1. Honey bee workers

The same source colonies of *Apis mellifera ligustica* used for the oral exposure were used for assessment of the toxicokinetics following contact acute exposure. As described in more detail in Medrzycki et al., 2021, newly emerged worker bees were incubated at 33 °C for 3-4 days, while being fed with sucrose solution *ad libitum*. Bees were then randomly allocated to the test cages (30 bees per cage) after being anaesthetised by CO2:air mixture for a short time interval. One cage per time point per replicate was set up, and three replicates were performed. 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 5ng of sulfoxaflor, 1µg of azoxystrobin, or 1µg of glyphosate. The solutions were prepared with sulfoxaflor and azoxystrobin in pure form, while glyphosate was contained in the commercial formulation Roundup Platinum.

After treatment bees were then incubated and fed *ad libitum* with sucrose solution, apart from the sampling points, where the bees were starved for 2 hours before freezing the whole test cage at -18°C. The initial level corresponds to immediately after exposure, while the final level corresponds to 96 hours after exposure for sulfoxaflor and to 7 days after exposure for azoxystrobin and glyphosate.

Table 8: Degradation of the active substance in worker honey bees exposed by contact.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	0.065	0.013	80%
Azoxystrobin	2.362	0.383	84%
Glyphosate (Roundup Platinum)	0.265	0.231	11%

Results show that in honey bee workers degradation of sulfoxaflor and azoxystrobin was higher after contact, when compared with oral exposure, while for glyphosate degradation was higher in the oral exposure (52%).

3.1.2. Honey bee queens

The same source of honey bee queens used for the oral exposure were used for topic exposure. Similar to workers, honey bee queens were individually treated with the spiked test solutions. Results are summarized in Table 9.

Table 9: Degradation of the active substance in queen honey bees exposed by contact.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	0.018	<0.010	99%
Azoxystrobin	13.920	2.997	78%
Glyphosate (Roundup Platinum)	0.140	0.163	0%

The type of exposure influenced degradation in opposite ways: for sulfoxaflor, following topic exposure degradation was highest; for glyphosate, 24 hours post topic exposure, the level of the a.i. was unchanged.

3.1.3. Honey bee drones

Honey bee drones were tested using the same methods as for honey bee workers. Results are summarized in Table 10. Initial levels are immediately after exposure while final levels were measured 96h post exposure for sulfoxaflor and 10 days after exposure for azoxystrobin and glyphosate.

Table 10: Degradation of the active substance in drone honey bees exposed by contact.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	0.024	0.010	58%
Azoxystrobin	3.965	0.273	93%
Glyphosate (Roundup Platinum)	3.269	0.409	87%

Degradation in drones was over 50% for all tested a.i. and highest for azoxystrobin, similar to workers. Glyphosate showed highest degradation in drones, compared to workers and queens.

3.2. Bumble bees

The toxicological endpoints and metabolization rate of agrochemicals after contact exposure on workers, males and queens of *Bombus terrestris* were determined.

The protocols for bumble bees were designed based on the official existing guidelines edited by OECD (2017) and are detailed in D3.2 (Medrzycki, P. *et al*. 2021). For contact exposure, bees were dosed individually with one of the chemicals sulfoxaflor or azoxystrobin in pure form. Glyphosate was tested as commercial formulation ROUNDUP FLEX which contains 450g of active ingredient per litre of formulation. The initial level for sulfoxaflor and azoxystrobin is the amount of residues in samples collected immediately after exposure, while the final level is residues in samples collected 72 hours after exposure. In glyphosate tests, the initial level is the amount of residues in samples collected 2 hours after exposure, while the final level residues on samples collected 48 hours after exposure. Levels of residues found in the bumble bee samples at the beginning and at the end of the experiment are reported in Tables 8, 9 and 10.

3.2.1. Bumble bee workers

Each worker bumble bee was treated with 1 µg of sulfoxaflor, 100 µg of azoxystrobin and 200 µg of glyphosate. The treatment dose volume was a 2 µl drop on thorax.

Table 11: Degradation of the active substance in worker bumble bees exposed by contact.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	10.1	3.71	63%
Azoxystrobin	760	188	75%
Glyphosate	0.468	0.287	39%

Degradation 72 hours after exposure of bumble bee workers was highest for azoxystrobin and lowest for glyphosate.

3.2.2. Bumble bee males

Each male bumble bee was treated with 0.5µg of the sulfoxaflor, 100 µg of azoxystrobin and 200 µg of glyphosate. The treatment dose volume was a 2 µl drop on thorax.

Table 12: Degradation of the active substance in male bumble bees exposed by contact.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	5.887	1.237	79%
Azoxystrobin	1218.601	536.466	56%
Glyphosate	0.391	0.194	50%

Degradation 72 hours after exposure of bumble bee males was highest for sulfoxaflor, and in any case 50% or higher for glyphosate and azoxystrobin.

3.2.3. Bumble bee queens

Each bumble bee queen was treated 20 µg of sulfoxaflor, 100 µg of azoxystrobin and 200 µg of glyphosate. The treatment volume dose was a 4 µl drop on thorax.

Table 13: Degradation of the active substance in queen bumble bees exposed by contact.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	38.232	28.423	26%
Azoxystrobin	123.337	25.104	80%
Glyphosate	0.193	0.097	50%

Degradation 72 hours after exposure of bumble bee queens was highest for azoxystrobin and lowest for sulfoxaflor.

3.3. Solitary bee (*Osmia bicornis*)

The methods used for contact exposure of *O. bicornis* are described in detail in Medrzycki, P. *et al*. (2021). The food source given during testing was *ad libitum* 50% w/v sucrose solution. The nominal doses given were sulfoxaflor 0.00313 µg/bee (dissolved in acetone), azoxystrobin 1µg/bee (dissolved in acetone), and glyphosate (RoundUp ProActive, Bayer, Germany, diluted in water and with 0.01% TritonX added as surfactant) given in a 1µL droplet. At least 1 g 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. The residues per bee at the first and final sampling points are presented in Tables 14 and 15.

Table 14: Degradation of the active substances in male *O. bicornis*, exposed by contact.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	0.385	0.118	68%
Azoxystrobin	11.315	1.391	87%
Glyphosate	16.346	0.939	94%

Table 15: Degradation of the active substances in female *O. bicornis*, exposed by contact.

Substance	Initial level (µg/g)	Final level (µg/g)	Degradation rate
Sulfoxaflor	0.082	0.064	21%
Azoxystrobin	6.838	2.115	69%
Glyphosate	6.252	1.226	80%

The results show a considerably lower degradation rate of azoxystrobin after 8 days when exposed by contact (87-69%) compared to oral exposure (>99%). For sulfoxaflor, the degradation rates after 48 h are slower when contact exposed (21-68%) compared to orally exposed (67-71%). Glyphosate degrades faster with contact exposure (80-94%) compared with oral (41-71%).

3.4. Comparison of toxicokinetics across species, sexes, castes and exposure modes

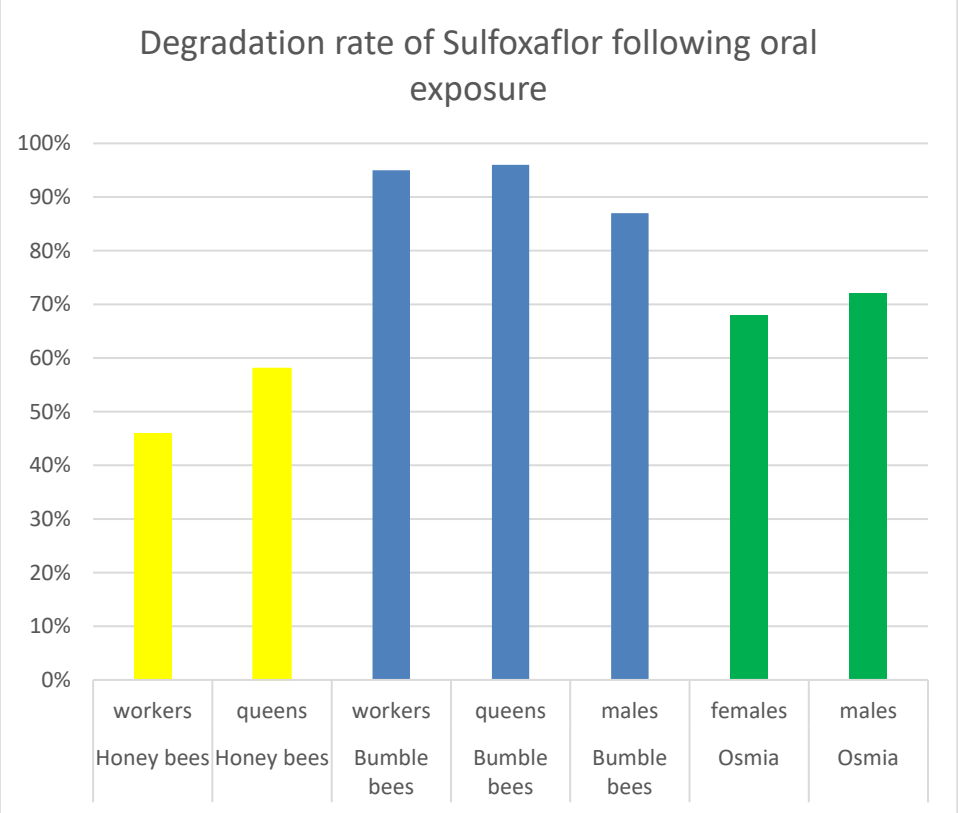

Figure 1: Degradation rate of sulfoxaflor following oral exposure in worker and queen honey bees (final level 72h and 24h post exposure respectively), worker, queen and male bumble bees (72h post exposure) and female and male *O. bicornis* (48h post exposure).

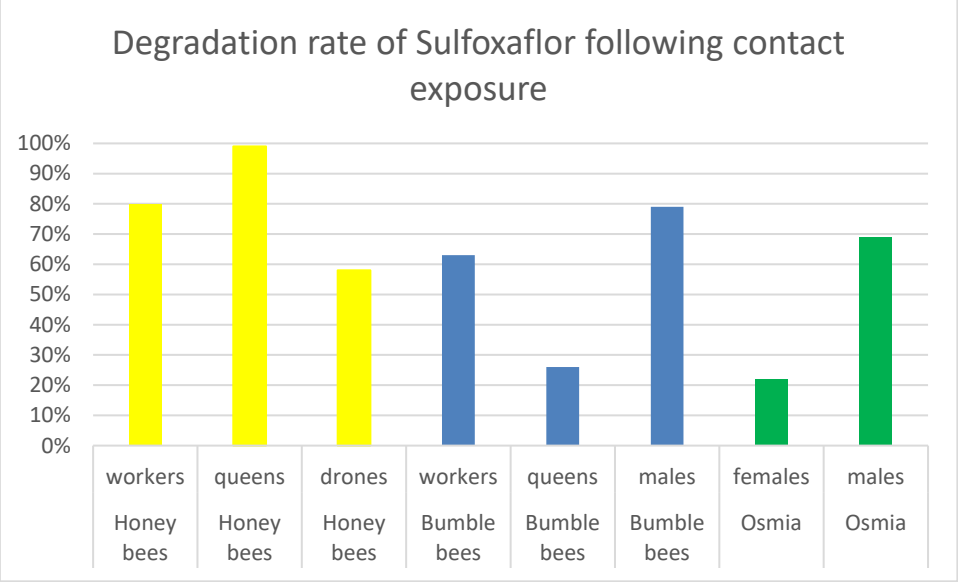

Figure 2: Degradation rate of sulfoxaflor following contact exposure in worker and drone honey bees (final level 96h post exposure) and in honey bee queens (final level 24h post exposure), worker, male and queen bumble bees (72h post exposure) and male and female *O. bicornis* (48h post exposure).

Degradation of sulfoxaflor in bumble bees and *Osmia* females was higher when the bees were orally exposed to the active ingredient. Conversely, in honey bee workers sulfoxaflor degraded at a higher rate when insects were topically exposed. A high variability of degradation rates was observed (Figure 1 and Figure 2).

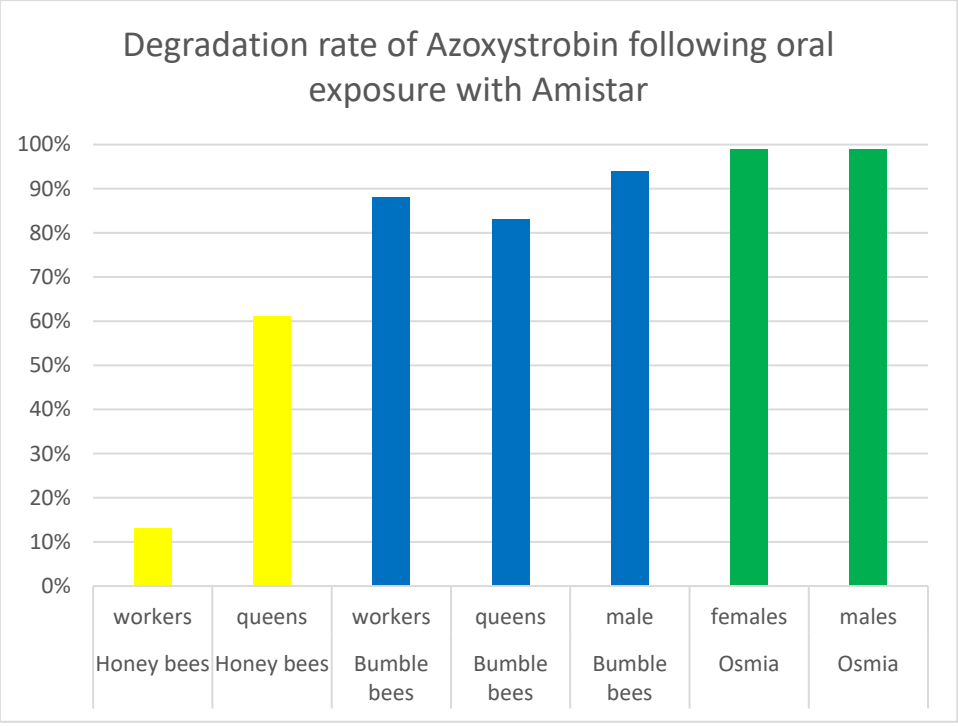

Figure 3: Degradation rate of Azoxystrobin following oral exposure in worker and queen honey bees (final level 10d and 24h post exposure respectively), worker, queen and male bumble bees (72h post exposure) and male and female *O. bicornis* (8d post exposure).

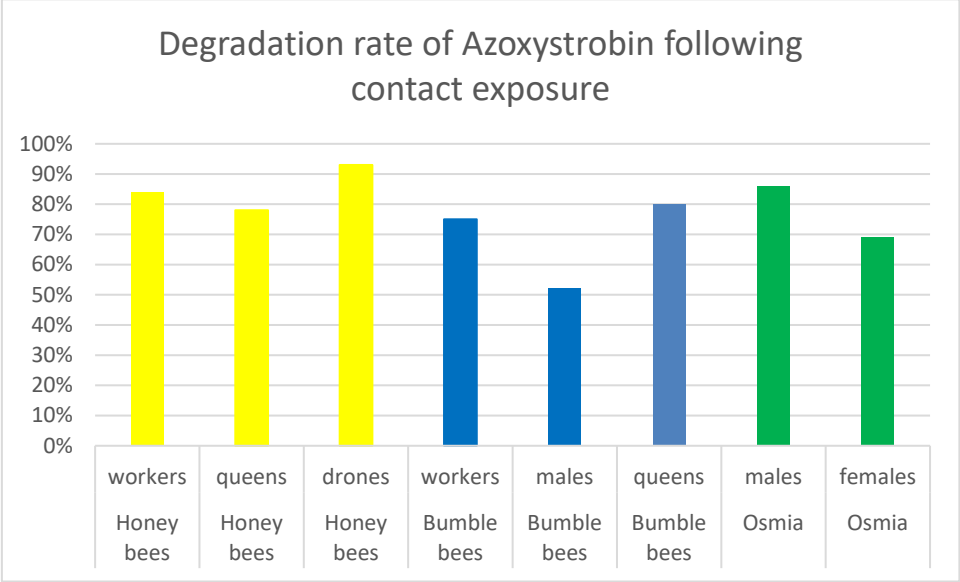

Figure 4: Degradation rate of azoxystrobin following contact exposure in worker, queen and dronehoney bees (final levels 7d, 24h and 10d post exposure, respectively), worker, male and queen bumble bees (72h post exposure) and male and female *O. bicornis* (8d post exposure).

Degradation of azoxystrobin was found to be lowest in worker honey bees exposed orally (Figure 3), different from what was observed in bumble bees and mason bees where degradation rate in the considered time spans was over 80% (almost 100% for mason bees). In honey bee queens degradation of azoxystrobin administered orally was notably higher than in workers. Degradation in bees topically exposed appeared to be more variable among sexes and species (Figure 4).

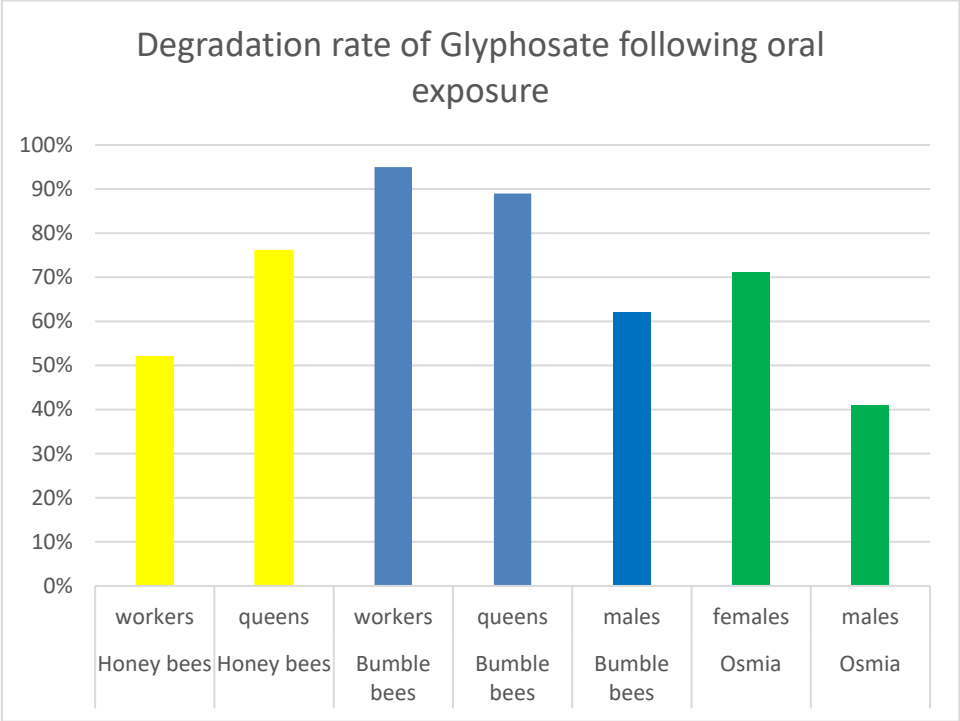

Figure 5: Degradation rate of glyphosate following oral exposure in worker, queen and drone honey bees (final levels 10d and 24h post exposure, respectively), worker, male and queen bumble bees (72h post exposure) and male and female *O. bicornis* (14d post exposure).

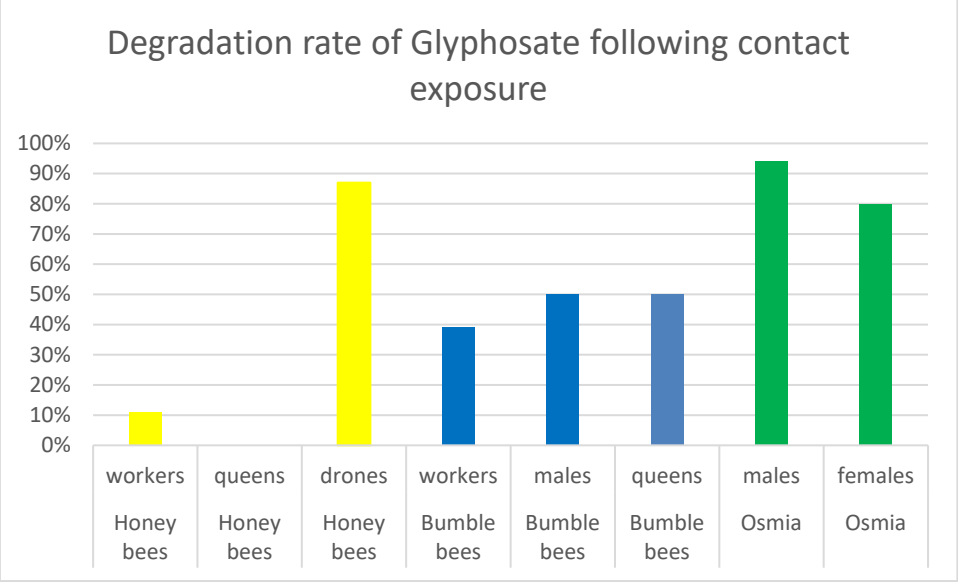

Figure 6: Degradation rate of glyphosate following contact exposure in worker, queen and drone honey bees (final levels 7d, 24h and 10d post exposure, respectively), worker, male and queen bumble bees (48h post exposure) and male and female *O. bicornis* (14d post exposure).

Degradation of glyphosate in the considered time intervals was lowest in worker and queen honey bees exposed topically. For honey bees and bumble bees degradation was higher after oral exposure, while in mason bees degradation was higher after topical exposure

3.5. Conclusions

Degradation of the tested substances showed great variation across species and exposure modes. In honey bees, degradation was highest in queens topically exposed to sulfoxaflor (99%) and lowest in queens topically exposed to glyphosate. Following oral exposure, in honey bees degradation was highest in queens, for all a. i., although the chosen time interval was only 24 hours for queens and minimum 72 hours for workers. In honey bees oral exposure seemed to cause a lower breakdown of the active ingredients sulfoxaflor and azoxystrobin. In bees exposed orally to azoxystrobin (administered as commercial formulation AMISTAR) and topically to glyphosate (administered as commercial formulation ROUNDUP PLATINUM) the a.i. seemed to remain stable in the bee body (degradation rates respectively 13% and 11%). This could be due to accumulation of the substances in the fat bodies, although specific studies with separation of the different bee organs should be performed to confirm this.

In bumble bees degradation rates were more uniform and on average around 70% for all tested substances. Queen bumble bees showed a much lower degradation of sulfoxaflor following contact exposure (26% versus 96% after oral exposure), and in general the results indicate similar dynamics of breakdown, with higher degradation occurring after oral exposure for all tested substances. In workers and queens all three substances administered orally were almost completely degraded 72 h after exposure.

When looking at the breakdown dynamics in *Osmia*, it appears that detoxification of sulfoxaflor is slower following contact exposure in comparison to oral exposure. This difference was noticed in females (22% degradation rate). Another sex-dependent difference was noticed for glyphosate administered orally, which remained more stable in Osmia males compared to females (41% versus 71%). Azoxystrobin breakdown times appear to be different between exposure routes, with breakdown being faster when exposed orally, compared to contact. 14 days after exposure 99% of

the substance initially present had disappeared in *O. bicornis* exposed orally, while in the *O. bicornis* exposed by contact there were still detectable levels of residues.

It should be noted that the difference in metabolism rate may be due to the pure compound being used for contact exposure vs the formulation AMISTAR in oral exposure. The decision to use the commercial formulation was due to the pure compound's insolubility in sugar syrup. This may confound the findings, since the presence of formulants may affect the metabolization and breakdown time of the active ingredient.

Overall we can conclude that metabolization of the active ingredients differs between species, castes and sexes, and is also affected by the exposure route.

The results of these experiments provide new insights into the dynamics of agrochemical breakdown and systemic exposure in individual bees, and indicate directions for further studies to better comprehend the relationship between degradation rates and toxicity.

4. Acknowledgements

Chemical analyses for detection of residues of azoxystrobin and sulfoxaflor were performed by the CREA-AA honey bee products laboratory (https://www.crea.gov.it/en/web/agricoltura-e-ambiente/-/specialised-analyses-performed-by-accredited-laboratory-accredia-0196-conforming-to-uni-cei-en-iso-iec-17025) while analyses for detection of glyphosate residues were performed by Lifeanalytics Srl, Honey Competence Centre (https://www.lifeanalytics.it/lifeanalytics-cuneo/).

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5. References

Aizen, M.A. and Harder, L.D. (2009) The Global Stock of Domesticated Honey Bees Is Growing Slower than Agricultural Demand for Pollination. Current Biology, 19, 915-918.

Brodschneider, Gray, van der Zee, Adjlane, Brusbardis, et al (2016) Preliminary analysis of loss rates of honey bee colonies during winter 2015/16 from the COLOSS survey. Journal of Apicultural Research, 55: 375-378. DOI:10.1080/00218839.2016.1260240

Garibaldi, L.A., Steffan-Dewenter, I., Winfree, R., Aizen, M.A., Bommarco, R. et al (2013) Wild pollinators enhance fruit set of crops regardless of honey bee abundance. Science, 339, 1608-1611.

IPBES (2016). The assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food production. S.G. Potts, V. L. Imperatriz-Fonseca, and H. T. Ngo (eds). Secretariat of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services, Bonn, Germany. 552 pages.

Klein, A.M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C. and Tscharntke, T. (2007) Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B-Biological Sciences, 274, 303-313.

Medrzycki, P., Hellström, S., Straw,E., Linguadoca, A., Jürison, M., Alaux, C.*et al*. (2021). Improved protocols for testing agrochemicals in bees. Deliverable D3.2. EU Horizon 2020 PoshBee Project, Grant agreement No. 77392

Meixner, M., Costa, C., Kryger, P., Hatjina, F., Bouga, M., Ivanova, E., Buechler, R. (2010) Conserving diversity and vitality for honey bee breeding. Journal of Apicultural Research, 49(1): 85-92. DOI 10.3896/IBRA.1.49.1.12

OECD (2017). OECD 246 Guideline for The Testing Of Chemicals Bumblebee, Acute Contact Toxicity Test.

Potts, S., Imperatriz-Fonseca, V., Ngo, H. et al. Safeguarding pollinators and their values to human well-being. Nature 540, 220–229 (2016). https://doi.org/10.1038/nature20588