グリホサートカリウム塩

要旨及び評価結果

（生活環境動植物及び家畜に対する毒性）

検索期間：2020 年 7 月 1 日～2020 年 12 月 31 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 8.2.2 and CA 8.2.2.1 and CP 10.2.2
Report author	Forner-Piquer I. *et al.*
Report year	2021
Report title	Differential impact of dose-range glyphosate on locomotor behavior, neuronal activity, glio-cerebrovascular structures, and transcript regulations in zebrafish larvae.
Document No	Chemosphere (2021), Vol. 267, Art. No. 128986
Guidelines followed in study	OECD TG 236 partially
Deviations from current test guideline	Deviations from OECD TG 236: • Indicators of lethality as coagulation of fertilised eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac and lack of heartbeat not reported as recommended in the OECD TG, but growth, cardiac and brain edema, general and brain necrosis, curled tail, pigmentation, tail fin, notochord, eyes, optic capsule and muscles indicators are reported instead. • Validity criteria of overall fertilisation rate, water temperature in test chambers, exposure to positive control and dissolved oxygen at the end of the 96 hrs exposure are not reported. • Apical observations of embryos 24 - 96 hrs post fertilisation are not reported • Water quality (pH, total hardness and conductivity in the control) is not reported • Glyphosate concentration in the lowest test concentration is not reported • Tested concentrations are not spaced by a factor not exceeding 2.2
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

Zebrafish (*Danio rerio*) larvae were exposed to glyphosate concentrations between 0.05 and 10.000 µg/L from 1.5 to 120 h post fertilization (hpf). Mortality was assessed at 24 hpf and cumulative hatching at 72 and 96 hpf. Gross morphology parameters were recorded until 120 hpf. The exposure of zebrafish to glyphosate concentrations had no increasing effect on mortality after 24 hpf. No significant effects were observed at the hatching rates at 72 hpf and 96 hpf and for morphological differences at 120 hpf. Zebrafish larvae showed behavioural changes in locomotor activity at concentrations equal and higher 1000 µg a.s./L. No significant behavioural changes were observed at concentrations lower than 10 µg/L.

Materials and methods

Test item: Glyphosate (N-(Phosphonomethyl) glycine); Sigma–Aldrich; CAS Number 1071-83-6; content of active substance (AS) 98.5%.

Test species: Zebrafish (*Danio rerio*); wild type AB strain.

Test design: Glyphosate was diluted in water-based E3 medium to obtain the requested concentrations. Zebrafish larvae were exposed to 8 concentrations of glyphosate: 0.05, 0.1, 0.5, 1, 10, 100, 1000, 10.000 µg/L from 1.5 to 120 h post fertilization (hpf). Solutions were renewed every day.

Mortality was assessed at 24 hpf and cumulative hatching at 72 and 96 hpf. Gross morphology was observed daily (e.g. growth, necrosis, eyes) until 120 hpf, using a stereomicroscope. Standard length was measured daily. Swimming bladder area, eye diameter and head-trunk angle were assessed at 120 hpf.

Locomotor behavioural activity like total distance travelled and velocity were assessed and recorded in a dark environment in an observation chamber coupled with video tracking. Therefore, all larvae at 120 hpf were transferred to a multi-well plate and acclimatized, for one hour and additionally for further 3 minutes after re-positioning in an incubator at 28°C and darkness, prior testing. Each treatment consists of 2 replicate plates with 24 larvae each plate. Locomotor behavioural activity data were smoothed with a Minimal Distance Moved threshold of 0.2 mm and with a Maximum Distance Moved filter of 8 mm to exclude small movements.

Glyphosate concentrations were analysed using liquid chromatography-mass spectrometry (LC-MS/MS).

Statistical analysis of morphology data and behavioural changes in locomotor activity was carried out with one-way ANOVA followed by Dunnett's multiple comparisons test. Cumulative hatching rate was analysed using two-way ANOVA followed by Dunnett's multiple comparisons test ($p < 0.05$).

Results

Analytical results (from supplementary data)

Analysis of glyphosate concentration was limited to high concentrations because of the limit of quantification of the technique used (10 µg/L). In particular, nominal levels of 1000 and 10.000 µg/L corresponded to actual values of 980 ± 310 µg/L and 6800 ± 1600 µg/L respectively.

Biological results

Table 1: Hatching rates and gross morphological parameters of zebrafish exposed to glyphosate

Treatment (µg a.s./L)	Mean hatching rate ± SD [a]		Mean total length ± SD (µm)	Mean swim bladder area ± SD ($µm^2$)	Mean eye diameter ± SD (µm)	Mean trunk - head angle ± SD (deg)
	72 hpf	96 hpf				
Control	73.20 ± 16.70	98.50 ± 2.60	3717 ± 89.3	61955 ± 4388	340.2 ± 18.0	157.3 ± 4.6
0.05	46.1 ± 7.70	99.30 ± 1.20	3696 ± 94.2	63331 ± 3172	325.1 ± 11.5	155.4 ± 2.9
0.1	63.80 ± 15.50	100 ± 0.00	3730 ± 67.2	63023 ± 6547	324.9 ± 18.5	156.0 ± 3.3
0.5	51.50 ± 20.30	100 ± 0.00	3730 ± 67.0	62653 ± 4355	324.8 ± 18.5	155.7 ± 2.10
1	63.80 ± 15.50	97.80 ± 3.90	3710 ± 40.0	61320 ± 5652	325.2 ± 15.6	156.8 ± 2.6
10	51.50 ± 20.30	100 ± 0.00	3676 ± 112.9	61930 ± 3973	323.5 ± 12.0	155.4 ± 2.3
100	39.30 ± 14.80	94.80 ± 5.10	3738 ± 109.3	62309 ± 2714	315.6 ± 12.1	155.5 ± 1.9
1,000	50.40 ± 23.90	98.70 ± 1.20	3736 ± 59.1	62089 ± 5004	325.0 ± 28.4	154.5 ± 2.7
10,000	62.10 ± 26.40	97.60 ± 2.50	3762 ± 62.9	64713 ± 4389	332.7 ± 8.42	157.0 ± 3.3

hpf Hours post fertilization
SD Standard deviation
[a] Expressed as hatched eggs/total x 100
deg Degree

No increase in mortality was observed following exposure to glyphosate (data not shown).

A trend decrease of hatching rates was observed at 72 hpf, although by 96 hpf no difference occurred.

No significant morphological differences were observed at any of the glyphosate concentrations tested.

All locomotor behavioural activities, observed at 120 hpf, showed significant effects at glyphosate concentrations equal or higher than 1000 µg/L compared to the control. Especially distance, mean velocity, number of rotations, and body mobility were reduced. No significant behavioural changes were observed at concentrations lower than 10 µg/L.

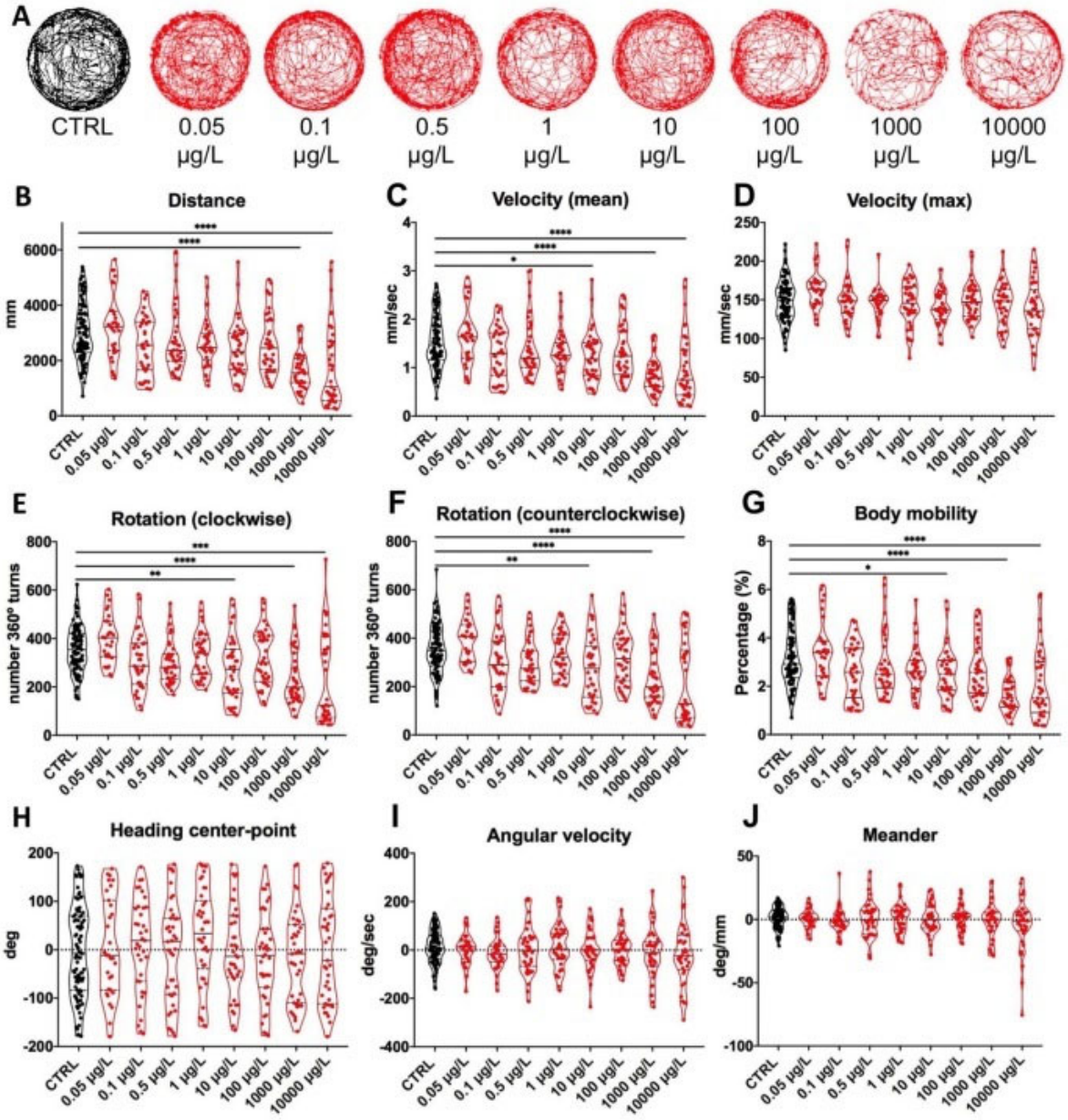

Figure 1: Behavioural defects in zebrafish larvae elicit with increasing glyphosate concentrations. A) Examples of 30-min swimming paths for each experimental group. B) Distance in mm. C) Mean velocity in mm/s. D) Maximum velocity in mm/s. E) Clockwise rotation in number of 360 turns. F) Counter-clockwise rotation in number of 360 turns. G) Percentage of body mobility. H) Direction of the body in degrees I) Angular velocity in degrees/s. J) Convolution of the movement in degrees/mm. Data are reported as mean ± SD, (1-way ANOVA, Dunnett's multiple comparison test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Experiments were performed in duplicate (CTRL n = 90, glyphosate groups n = 40).

Conclusion

The exposure of zebrafish to glyphosate concentrations had no increasing effect on mortality after 24 hpf. No significant effects were observed at the hatching rates at 72 hpf and 96 hpf and for morphological differences at 120 hpf.

The effect of glyphosate on zebrafish larvae showed behavioral changes in locomotor activity at concentrations equal and higher 1000 µg a.s./L. No significant behavioural changes were observed at concentrations lower than 10 µg/L.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Zebrafish (*Danio rerio*) larvae were exposed to glyphosate concentrations between 0.05 and 10.000 µg/L from 1.5 to 120 h post fertilization (hpf). Mortality, cumulative hatching rate and morphological changes showed no significant effects at any glyphosate concentration. Behavioural changes in locomotor were observed at concentrations equal and higher 1000 µg a.s./L. No significant behavioural changes were observed at concentrations lower than 10 µg/L.

A wide range of concentrations (8 between 0.05 and 10.000 µg/L) were tested, which does not allow the estimation of EC_xs. Not all validity criteria according to OECD TG 236 (Fish Embryo Acute Toxicity Test) can be evaluated. Analytical verifications of the test concentrations and some methodological data are not fully reported. In addition, water quality parameters were not reported. The study is partially compliant with the OECD TG 236: Fish Embryo Acute Toxicity Test. Several LOEC and/or NOEC (hatching rate, morphological and behavioural parameters, etc.) can be obtained from the study.

This study has been classified as relevant (Category A acc. EFSA GD 2092, Point 5.4.1) and reliable with restrictions.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 8.2.2 and CP 10.2.2
Report author	Du-Carree J.L. *et al.*
Report year	2021
Report title	Impact of chronic exposure of rainbow trout, *Oncorhynchus mykiss*, to low doses of glyphosate or glyphosate-based herbicides.
Document No	Aquatic toxicology (2021), Vol. 230, Art No. 105687
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions

2. Full summary of the study according to OECD format

The effects of direct and chronic exposure of glyphosate on the health status of the rainbow trout, *Oncorhynchus mykiss* was assessed for ten months. In a continuous flow-through test, 12 fishes were exposed daily to a dose of around 1 µg a.s./L for one hour with the water flow stopped and then set up at 135 L/hour for the rest of the day, allowing for the gradual dilution of glyphosate. Additionally, an unexposed control group was included. The effect on the reproductive capacities was analysed over a period of 4 months covering spawning.
No mortality was recorded before the spawning period. Spawning induced mortality rates between 15% and 30%, without significant differences between the treatment groups. Growth and reproduction showed no statistical differences between the glyphosate treatment and the control group. These results suggest that a 10 months exposure of rainbow trout to a daily mean theoretical concentration of 0.123 µg/L of glyphosate administered using the pure active substance did not significantly modify their global health including during the spawning period.

Materials and methods

Test item: Glyphosate; Sigma-Aldrich; ref. 45521; CAS Number 1071-83-6; content of active substance (AS) 98%.

Test species: Rainbow trout (*Oncorhynchus mykiss*); three-year-old specific pathogen free (SPF) males and females from the protected and monitored fish facilities of the ANSES Plouzané Laboratory site (France).

Test conditions: In 400 L water tanks, fish were maintained under continuous flow-through conditions (300 L/h) and oxygen levels above 60% of saturation with aeration. Fish were fed daily at 1.5% of the biomass and submitted to a natural photoperiod and a temperature increasing from 9 to 21ºC between April and July and decreasing from 21 to 8ºC between July and November. Eggs during embryonic development were maintained at 8 ± 2°C.

Test design: 12 fish (male/female proportion of 1:2) per test item and control treatment were exposed every working day to 100 mL of a prepared concentrated glyphosate solution of 4 mg a.s./L, resulting in around 1 µg a.s./L of exposure. During exposure, the continuous flow-through (300 L/h) was stopped for one hour, so that the fish get in contact with the test substance. Afterwards, the flow-through was set up at 135 L/h for the rest of the day for a gradual dilution of glyphosate. Taking theoretical kinetics of glyphosate concentrations in contaminated tanks into account, the integrated mean theoretical concentration of glyphosate for each contamination day was approx. 123 ng/L.

The spawning period occurred in November 2018 and the maturation of fish was tested every week by exerting manual abdominal stripping to ensure spawning on a day when most of the fish were mature. Stripping events were done twice over the spawning period to collect eggs and sperm from the most mature fish, with approximately two weeks between them. In each treatment, sperm of all mature males were used to fertilize eggs of mature females. Females were weighed before and after stripping to measure the total weight of eggs. A fraction of eggs for each mature female was sampled for egg numbering and weighing. Relative fecundity was considered as the number of eggs divided by the weight of each respective female after stripping.

The embryonic development was conducted in tank containing approximately 300 L continuously renewed with a river water flow (approximately 300 L/h), after fertilising the eggs of each female with pooled sperm from each treatment. For the control four females and for glyphosate seven females were considered. Fertility was considered as the proportion of eggs surviving at five days post fertilization. Survival of eggs was assessed daily for each female on a fraction of approximately 200 eggs isolated in plastic breeding boxes.

Water tank samples were analysed for the actual concentration of glyphosate at the beginning (after 1 month of contamination) and at the end (after 10 month of contamination) of the exposure period using ELISA method for the first and HPLC for the second sampling. The metabolite AMPA was also quantified by HPLC.

For statistical analysis, data were tested for normality and homoscedasticity. In the case of normal and homoscedastic data, one-way Anova tests were used to compare means, followed by a post-hoc test of Dunnett. In the case of normal and heteroscedastic data, modified one-way Anova tests were used to compare means, followed by a post-hoc test of Tamhane-Dunnett. In the case of non-normal data, a Kruskal-Wallis test was used to compare means, followed by a post-hoc test of Dunn. Differences between survival and fecundity percentages were compared with a Chi-squared test. A p-value of 0.05 was used as the threshold for statistical significance.

Results

Analytical results

Glyphosate was not detected in control tank throughout the experiment. The glyphosate metabolite AMPA was never detected in any of the exposure tanks. One hour after the addition of glyphosate, concentrations ranged between 1.05 and 1.45 µg a.s./L in all exposure tanks. The glyphosate concentration measured by HPLC was approx. 30-40% lower (0.77 µg a.s./L) at the same point of the kinetic. Two hours after restarting the water flow, glyphosate concentrations were similar (between 0.24 and 0.55 instead of the modelled concentration of 0.51 µg/L predicted by the theoretical dilution curve) regardless of the method used.

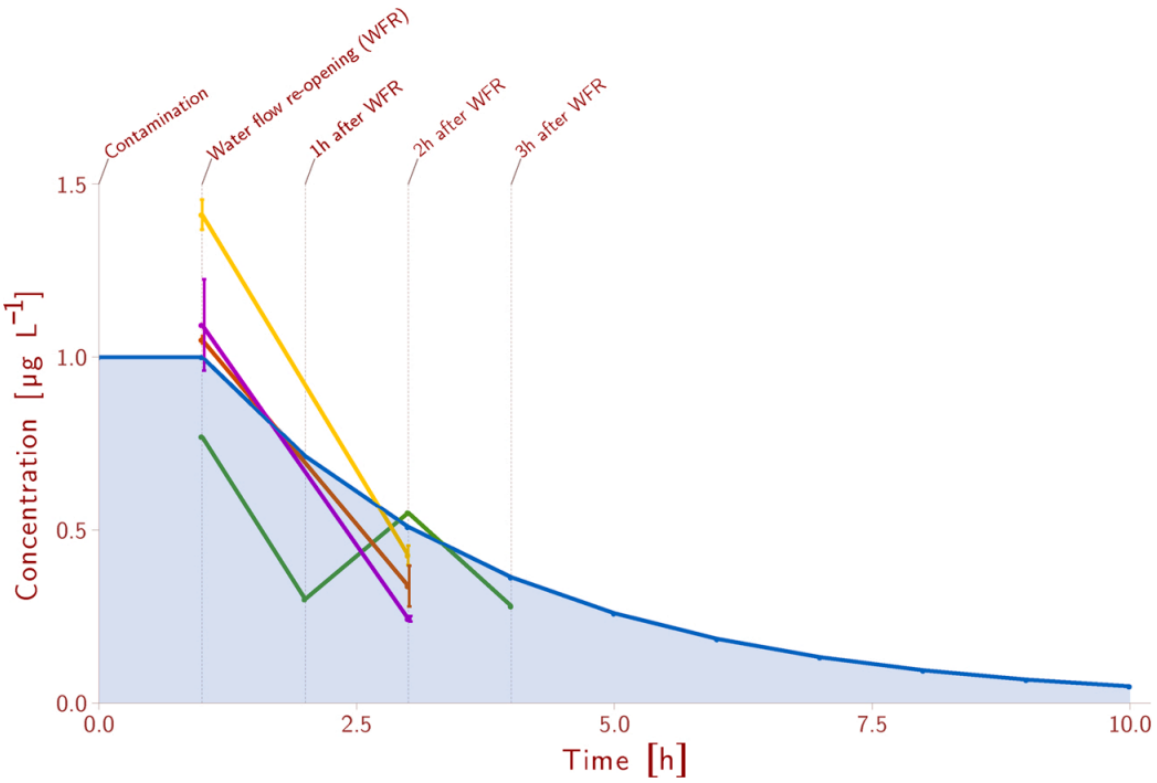

Figure 1: Mean concentration of glyphosate as a function of time (µg/L). Theoretical concentrations (in blue) are compared to concentrations measured at different times of the kinetic using ELISA or HPLC methods. ELISA analysis was done after 1 month of contamination for each condition (Glyphosate – G in yellow) and two points of the kinetics. HPLC analysis was done after 10 months of contamination in the tank contaminated with G for all points of the kinetics (in black). Error bars represent standard-errors.

Biological results

No mortality was recorded before the spawning period. Spawning induced mortality rates between 15% and 30% (data not shown), without significant differences between the treatment groups.

Outside of the spawning period, mortality events occurred in control and contaminated groups but no abnormal behaviour or clinical signs were observed.

Mean ratios of weights before and after the spawning period were 1.14 and 1.11 for the control and glyphosate, respectively. No statistical differences were found among the means of the treatment groups.

Table 1: Mean weights of mature rainbow trouts before and after spawning period

Treatment	**Mean weight ± SD (g)**		
	Before spawning [a]	**After spawning [b]**	**Ratios before/after**
Control	1208 ± 117	1387 ± 213	1.14 ± 0.13
Glyphosate 1 µg a.s./L	1131 ± 193	1249 ± 222	1.11 ± 0.11

SD Standard deviation
[a] measured in September 2018
[b] measured in November 2018

High fertility was observed for female rainbow trout in both treatments. Relative fecundity and fertility between control and contaminated fish were not significantly different.

Table 2: Mean reproductive parameters of female rainbow trout

Treatment	Reproduction	
	Relative fecundity ± SE (eggs/g fish)	**Fertility (%)**
Control	2.63 ± 0.72	99.6 ± 0.4
Glyphosate 1 µg a.s./L	2.71 ± 0.30	95.8 ± 3.9

SE Standard error

Conclusion

The effect of low and environmentally relevant concentrations of glyphosate on rainbow trout was tested in a long-term exposure. No mortality was recorded before the spawning period. Spawning induced mortality rates between 15% and 30%, without significant differences between the treatment groups. Growth and reproduction showed no statistical differences between the glyphosate treatment and the control group.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Rainbow trouts were exposed daily to glyphosate (1 µg a.s./L) for 10 month under flow-through conditions. Mortality, reproduction and growth showed no statistical differences between the glyphosate treatment and the control group.

In this study only one concentration was tested (1 µg a.s./L; low-dose). The characteristics and composition of the media used (water) were not fully described. The data on the technical material supports low chronic exposure risk. The test design is such that the fish were too big when used in the study and there is some uncertainty over the influence of the body size on the outcome of the study.

This study has been classified as relevant (Category A acc. EFSA GD 2092, Point 5.4.1) and reliable with restrictions.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 8.3.1.4, CP 10.3.1.5, CP 10.3.1.6
Report author	Odemer R. *et al.*
Report year	2020
Report title	Chronic High Glyphosate Exposure Delays Individual Worker Bee (*Apis mellifera* L.) Development under Field Conditions.
Document No	Insects (2020), Vol. 11, No. 10, Art. No. 664
Guidelines followed in study	OECD TG 75 modified and partially followed in Experiment 1.
Deviations from current test guideline	Deviations to OECD TG 75: • The temperature during the field experiments is only reported as an average of 18.9 °C without knowing if the recommended range of min. 15°C and max. 30°C was kept, to enable a sufficient flight activity of the bees. • Relative humidity and rainfall is not reported for the field experiments. • An assessment of control performance and acceptability of the study has not been stated despite the study appearing to have been conducted according to recognized approaches e.g. OECD 75 semi-field and tunnel test and the Oomen (1992) brood feeding approaches.
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable without restrictions

2. Full summary of the study according to OECD format

In this study three field and one semi-field tests were conducted in Germany to assess the effect of glyphosate based herbicides (Glyfos Unkraut-Frei® 360 g a.i./L and Roundup® Power Flex 480 g a.i./L) to honey bees (*A. mellifera*). The field studies assessed the effect on the brood and colony development, adult survival, and overwintering success of honey bees, while residues were measured, whereas the semi-field study determined residues of glyphosate based herbicides in different bee relevant matrices.
For brood effects and survival, mini-hives housed in the "Kieler mating-nuc" system were orally exposed to two concentrations of Glyfos Unkraut-Frei® 360 g a.i./L (T1: 4.8 mg a.i./kg and T2: 137.6 mg a.i./kg feeding syrup) over a period of one brood cycle (21 days). Brood development and colony conditions were assessed after a modified OECD method (No. 75). On day 21, pooled samples of the stored syrup were collected from in-hive storage cells for residue analyses. For adult survival, weighed and labelled freshly emerged workers were introduced into non-contaminated mini-hives to monitor their life span for 25 consecutive days.
For colony conditions under field conditions, honey bee colonies were exposed during summer to flowering *Phacelia* sprayed with Roundup® Power Flex (480 g a.i./L) at a rate of 3.75 L/ha. The number of bees, brood cells, and stores (honey and pollen) were estimated on DAT-6 (20 July) before the exposure and DAT+15 (10 August) and DAT+57 (21 September) after the exposure. Beebread (stored pollen), food (stored syrup), and plant samples were taken at different time intervals for residue analyses.
For the overwintering success, honey bee colonies were fed with one concentration of Roundup® Power Flex 480 g a.i./L (5.439 mg a.i./kg feeding syrup) and were assessed on DAT-1 (1 October) before the exposure and DAT+43 (14 November) and DAT+170 (21 March) after the exposure, using the "Liebefeld method". Samples of stored food were collected from in-hive storage cells for residue analyses at different time intervals.

For the determination of GBH residues, honey bee colonies placed in tunnel tents were exposed to flowering *Phacelia* sprayed with Roundup® Power Flex (480 g a.i./L) at a rate of 3.75 L/ha. Stored food, pooled honey sac, pooled corbicular pollen, and pooled plant samples were taken at different time intervals after the treatment from all tents for residue analyses.
Feeding exposure revealed no significant differences between groups in colony condition and development as well as in brood development. Significantly increased brood termination (BFD+21) and significantly lower hatching weight of adult workers in the test item treatment T2, was assessed when compared to the control.With a hazard ratio (HR) of 0.93 for T1 and 1.43 for T2, the treated bees were not expressing higher mortality when compared to the control.
At the foliar application experiments glyphosate based herbicides did not significantly affect colony development but a matrix-dependent exposure gradient could be identified, which could be presented from high to low glyphosate residues, as follows: corbicular pollen > plants > honey sac > stored food/nectar.

Materials and methods

Experiment 1: Brood Development and Survival
Test item: Glyfos Unkraut-Frei® (Dr. Stähler, Köln, Germany); content of active ingredient (a.i.) 360 g glyphosate/L.
Tested concentration: Glyfos Unkraut-Frei® was directly mixed into 5 L of feeding syrup (Apiinvert, Südzucker GmbH, Mannheim, Germany) to achieve the desired concentrations of 0.090 g product/5 L for T1 and 2.610 g product/5 L for T2, corresponding to measured concentrations of 4.8 mg a.i./kg (T1) and 137.6 mg a.i./kg (T2) feeding syrup. The control was fed with untreated syrup, which was provided in a weekly interval like in all other groups. Chronic treatment in T1 and T2 was maintained for 21 consecutive days to cover a full worker-brood cycle with a total amount of 0.81 kg feeding syrup per hive, corresponded to a calculated amount of 3.859 mg (T1) and 111.428 mg (T2) glyphosate per hive, respectively.
Test species: Honey bee (*Apis mellifera L.)*; mini-hives originating from brood frames of two healthy donor colonies with no clinical symptoms of adult bee or brood diseases visible during inspection.
Location of the field site: Near Wurmberg, Germany (LAT 48.867414°, LON 8.808538°) between June and September (field phase). Within proximity of 250 m, no other hives were set up, whereas in an extended radius (>250 m) a sufficient number of unrelated colonies were located to provide enough drones for mating. At the present time, bees could forage on *Tilia* spp., *Cyanus segetum,* and other floral sources in the surroundings.
Test conditions: Natural field conditions with an average outdoor temperature of 18.9 °C and a precipitation of 53.97 L/m2. The incubator for brood development was set at 33 °C, 70% RH and total darkness.

Experiment 2: Field Exposure
Test item: Roundup® Power Flex (Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany); content of active ingredient (a.i.) 480 g glyphosate/L.
Tested concentration: Roundup® Power Flex (480 g glyphosate/L) was applied with a rate of 3.75 L/ha in 300 L water/ha on the flowering *Phacelia*. The control remained unsprayed.
Test species: Honey bee (*Apis mellifera L.)*; healthy and queen-right colonies with one hive body including ten combs and no clinical symptoms of adult bee or brood disease were visible; colonies were as homogenous as possible with approx. 15,000 bees per colony; queens originated from one breeding line.
Location of the field site: Near Braunschweig, Germany (Control plot: LAT 52.296415°, LON 10.437062°; treatment plot: LAT 52.202098°, LON 10.623331°) during July and August. Colonies were placed at the edge of two flowering *Phacelia tanacetifolia* plots.

Experiment 3: Overwintering
Test item: Roundup® Power Flex (Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany); content of active ingredient (a.i.) 480 g glyphosate/L.
Tested concentration: Roundup® Power Flex was directly mixed into 5 L of feeding syrup (1:1 water sugar, w/w, density: 1.2296) for each colony to achieve the desired nominal concentration of 8.13 mg

a.i./kg. The control was fed with untreated syrup. Chronic exposure in the Roundup® Power Flex treatment was maintained until the feeder was emptied. The average measured concentration of 5.439 mg a.i./kg in the feeding solution corresponded to a calculated total amount of 33.436 mg glyphosate per hive.
Test species: Honey bee (*Apis mellifera L.)*; healthy and queen-right colonies with one hive body including ten combs and no clinical symptoms of adult bee or brood disease were visible; colonies were as homogenous as possible with approx. 10,000 bees per colony; queens originated from one breeding line.
Location of the field site: Near Braunschweig, Germany (LAT 52.202098°, LON 10.623331°) between October and March.

Experiment 4: Determination of Glyphosate Based Herbicides Residues
Test item: Roundup® Power Flex (Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany); content of active ingredient (a.i.) 480 g glyphosate/L.
Tested concentration: Roundup® Power Flex (480 g glyphosate/L) was applied with a rate of 3.75 L/ha in 300 L water/ha on the flowering phacelia. The control was sprayed with water.
Test species: Honey bee (*Apis mellifera L.)*; healthy and queen-right colonies with one hive body including ten combs and no clinical symptoms of adult bee or brood disease were visible; colonies were as homogenous as possible with approx. 15,000 bees per colony; queens originated from one breeding line.
Location of the field site: Near Braunschweig, Germany (LAT 52.296415°, LON 10.437062°) during July and August.

Test design:

Experiment 1: Brood Development and Survival
In this field study, 19 mini-hives were established with about 800 worker bees. Subsequently, unmated sister queens were introduced, and the mini-hives were placed at a remote apiary for mating. Mini-hives were each housed in a Styrofoam box with four top-bars, a strip of a beeswax foundation attached to it and a feeder attached to it. After five weeks, the established hives showed all the relevant brood stages (eggs, larvae, and patches of sealed brood) and newly built combs.
Mini-hives of one treatment group, consisted of five replicate colonies, were orally exposed to sublethal concentrations of 4.8 mg glyphosate/kg (T1, low) and 137.6 mg glyphosate/kg (T2, high) over a period of one brood cycle (21 days). Additionally, an untreated control was included. One T2 mini-hive was discovered to be queenless shortly after the start of the experiment and was therefore removed (n: C = 5, T1 = 5, T2 = 4).
After the 21 days chronic exposure, one sealed brood comb containing brood cells ready to hatch was removed from the mini-hives of all groups and put together treatment-wise (five combs from control and T1, and four from T2) in an incubator. After 24 h, all hatched bees were pooled treatment-wise, and 30–40 young workers were collected randomly. After documenting the hatching weight bees were introduced into the remaining four mini-hives from a total of 19 and were divided equally according to their treatment to total numbers of 152 (control), 149 (T1), 141 (T2) introduced individuals per treatment. Survival was then monitored for 25 days.
For adult survival, freshly emerged workers from control and exposed colonies were marked with a coloured and numbered opalith plate on the thorax for identification and then introduced into non-contaminated mini-hives for 25 consecutive days of monitoring. Every two to three days all combs, including the inside of the hive, were photographed for the subsequent counting of the marked bees on a computer screen.
Hatching weight was documented by weighting the bees according to their treatment and respective pool, resulting in a total number (n) of control (50), T1 (38), and T2 (36).
Colony conditions and brood development were assessed after a modified OECD method No. 75.
Colony conditions: The total number of bees was estimated for each colony. In addition, the absolute hive weight was recorded at the time of the colony assessments. The assessments were performed shortly before the first application, DAT0, DAT+30 and DAT+56.
Brood development: For the colony condition assessment on DAT0, one or two brood combs were taken out of each replicate from the control, T1, and T2 (Brood area Fixing Day = BFD) groups. The

development of the bee brood was continuously assessed and followed. Approximately 100 cells per replicate containing eggs were selected, uniquely identified and pictures were taken from the comb-area containing eggs. From this evaluation, the brood termination rate was respectively calculated.

Experiment 2: Field Exposure
In this field study, twelve honey bee colonies were split into two groups with six replicates each control and treatment group. Eight days before application (Day after treatment = DAT-8) the colonies were placed to the field site.
Roundup® Power Flex was applied with a rate of 3.75 L/ha in 300 L water/ha on the flowering *Phacelia* (BBCH 64–65) with a field sprayer. The control remained unsprayed. Colonies from the treatment plot were migrated after nine days of exposure (DAT+8) to the control plot.
On DAT-6 (20 July) before the exposure and DAT+15 (10 August) and DAT+57 (21 September) after the exposure, respectively, the number of bees, brood cells, and stores (honey and pollen) were estimated.

Experiment 3: Overwintering
In this field study, bees were exposed to Roundup® Power Flex orally with treated feeding syrup provided in feeder.
On DAT-1 (1 October) before the exposure and DAT+43 (14 November) and DAT+170 (21 March) after the exposure, respectively, the number of bees, number of brood cells, and the number of stores were estimated.

Experiment 4: Determination of Glyphosate Based Herbicides Residues
In this semi-filed study, twelve honey bee colonies were split into two groups with three replicates each (control and treatment group). Queens were removed on DAT-14 to stimulate foraging in the tunnel tents. On the day of application (DAT0, 2 August), each colony was placed in a tunnel tent with an area of approximately 33.5 m2 on a flowering *P. tanacetifolia* plot. On the application day (DAT0) Roundup® Power Flex was applied with a rate of 3.75 L/ha in 300 L water/ha on the flowering *Phacelia* (BBCH 65) using a portable boom sprayer. The control was sprayed with water. Due to the loss of forage in the GBH-treated tents, all colonies were migrated after 13 days of exposure (DAT+13, 15 August) to a remote apiary.
A total of four stored food, two pooled honey sac, two pooled corbicular pollen, and four pooled plant samples were taken at different time intervals after the treatment from all tents for residue analyses. On DAT+5, plants could no longer be sampled due to the action of the herbicide.

<u>Residue analyses</u>

Experiment 1: Brood Development and Survival
For residue analyses of glyphosate, a sample of each feeding regime and the untreated feeding syrup was collected before the experiment and on day 21 pooled samples of the stored syrup from all groups were collected from in-hive storage cells.

Experiment 2: Field Exposure
For residue analyses, 12 beebread (stored pollen), 60 food (stored syrup), and 10 plant samples (whole plant except the roots) were taken at different time intervals before and after the treatment from both plot sites.

Experiment 3: Overwintering
For residue analyses, 66 samples of stored food were collected from in-hive storage cells from all groups at different time intervals.

Experiment 4: Determination of Glyphosate Based Herbicides Residues
For residue determination of glyphosate (GLY) and its metabolite aminomethylphosphonic acid (AMPA) in the samples, LC-MS/MS was used. The samples of all the experiments (1-4) were analysed together. A total of 176 samples were measured, including controls (experiment 1: six samples, experiment 2: 92 samples, experiment 3: 66 samples, experiment 4: 12 samples).

Statistic (for all experiments)
Statistical analysis of the Mortality in the mini-hives was evaluated with a Kaplan–Meier survival analysis (KM) and with log-rank tests (Cox–Mantel) using the "pairwise_survdiff" function.
Statistical analysis of the estimated number of bees and brood cells from all the colony condition assessments, the hatching weight and weight gain of the mini-hives as well as the brood termination rate, and the number of selected eggs from the BFD was carried out with a Shapiro–Wilk test for normal distribution. Normally distributed date were analysed with a one-way ANOVA and non-normally distributed data with a Kruskal-Wallis test to compare multiple experimental groups, respectively. Statistically significant results were further tested pairwise with a Student's t-test or Wilcoxon rank-sum test. All analyses were performed in R version 3.6.2 with a significance level of $\alpha = 0.05$ for all tests.

Results

Experiment 1: Brood Development and Survival
Survival of the bees showed no significant differences between the Glyfos Unkraut-Frei® treatments T1 and T2 and the control.
The hatching weight of adult workers in T2 was significantly lower (16.7% reduction) when compared to the control with median values of control: 0.14, T1: 0.13, and T2: 0.12 g.
In addition, a Cox proportional hazards model was applied to determine the hazard ratio (HR), displayed as a forest plot (Supplementary data). With an HR of 0.93 for T1 and 1.43 for T2, the treated bees were not expressing higher mortality when compared to the control (T1: $p = 0.73$, T2: $p = 0.051$, log-rank test).

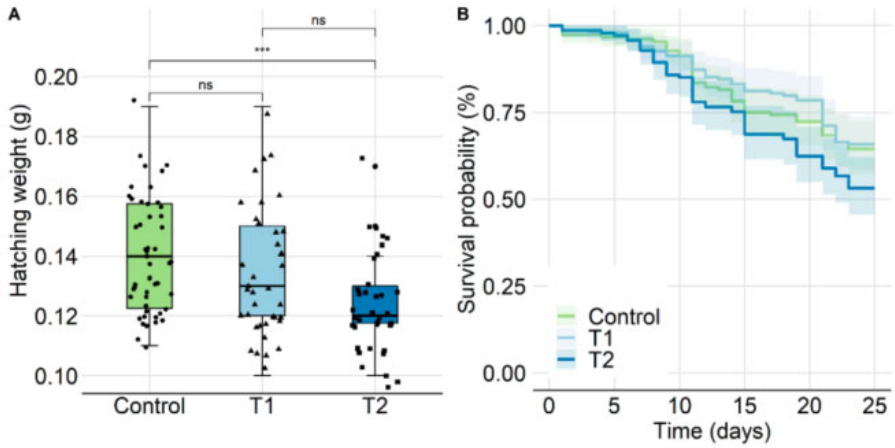

Figure 1. Effect of chronic Glyfos Unkraut-Frei® feeding exposure (T1: 4.8, T2: 137.6 mg a.i./kg) on hatching weight and survival of adult worker bees. (A) Hatching weight was assessed on the day of emergence after 24 h in the incubator and displayed as boxplot. Weight in T2 was significantly lower (16.7%) when compared to the control ($p < 0.01$, Wilcoxon rank-sum test, pairwise). (B) Survival of individual workers is illustrated with a Kaplan–Meier survival curve and 95% confidence intervals. A pairwise comparison with corrections for multiple testing did not confirm differences between the respective treatments and the control ($p > 0.05$, log-rank test, pairwise).

No significant effects in colony conditions between treatments and the control were observed.

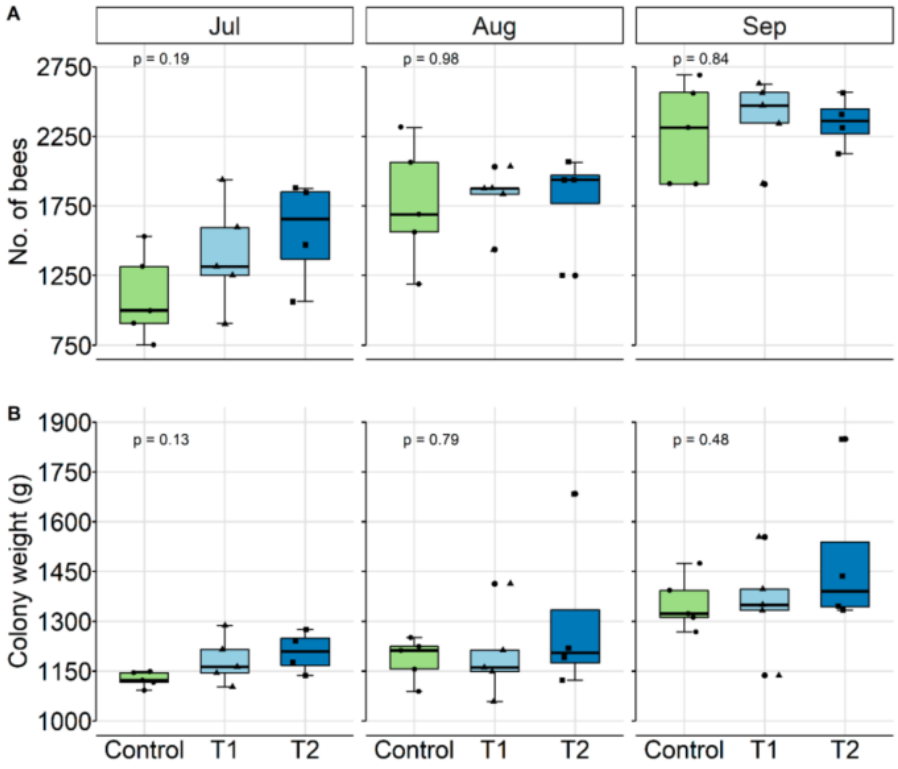

Figure 2. Effect of chronic Glyfos Unkraut-Frei® feeding exposure (T1: 4.8, T2: 137.6 mg a.i./kg) on colony conditions, displayed as a boxplot. (A) Number of worker bees increased similarly and was not significantly affected by the test item ($p > 0.05$, ANOVA). (B) Absolute colony weight and its increase during the study. There were no significant differences between groups ($p > 0.05$, Kruskal–Wallis test).

Successful brood development was observed in the control, T1, and T2 groups, in the majority of the marked brood cells. On BFD+13, the median termination rate was 13.04% in the control, 10.53% in T1 and 24.05% in T2. On BFD+21, the median termination rate was 22.11% in the control, 20.69% in T1 and 49.84% in T2. The T2, brood termination was significantly increased when compared to the control.

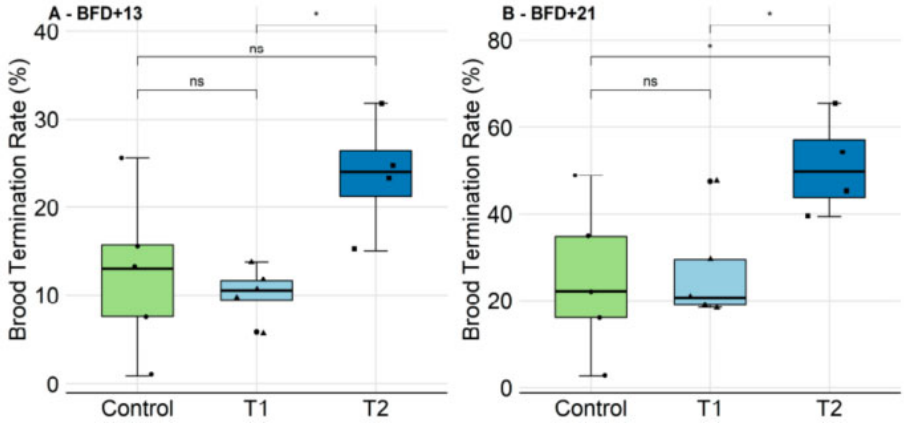

Figure 3. Effect of chronic Glyfos Unkraut-Frei® feeding exposure (T1: 4.8, T2: 137.6 mg a.i./kg) on the Brood Termination Rate (BTR) displayed as a boxplot. (A) The BTR of treatments T1 and T2 did not show significant differences when compared to the control ($p > 0.05$, t-test, pairwise) on BFD+13 (Brood area Fixing Day). (B) Eight days later, on BFD+21, T1 did not show differences when compared to the control ($p > 0.05$, t-test, pairwise). T2, however, revealed a significantly higher BTR ($p < 0.05$, t-test, pairwise).

Experiment 2: Field Exposure

Roundup® Power Flex did not significantly affect colony development. Bees and brood showed a pattern of increases and decreases in an alternate sequence. Brood cells were decreasing faster in the control on DAT+57 due to a greater winter food intake.

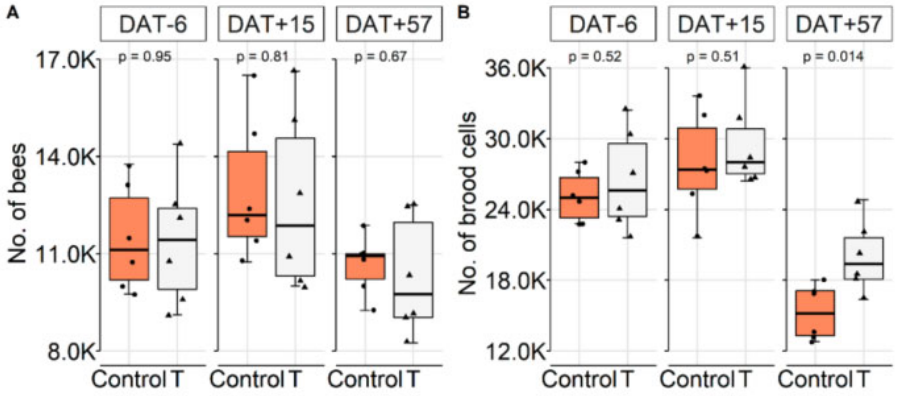

Figure 4. Effect of GBH field exposure (foliar spray) on colony conditions, displayed as boxplot. (A) Number of worker bees. (B) Number of brood cells during the study. On DAT+57, the treatment T had significantly more brood cells when compared to the control (Wilcoxon rank-sum test). For all other assessments, conditions were not significantly different. Dates correspond to the following months: DAT−6 (=20 July), DAT+15 (=10 August), and DAT+57 (=21 September).

Experiment 3: Overwintering

Shortly before hibernation (DAT+43), brood activity was reduced in all colonies independent of their treatment, followed by a continuous decrease of bees. At the end of overwintering, a decrease of stored food was observed (Supplementary data) as a result of the returning brood activity. Roundup® Power Flex did not significantly affect colony development. On DAT+170, we found that two colonies in the control and one in the treatment group had not survived winter.

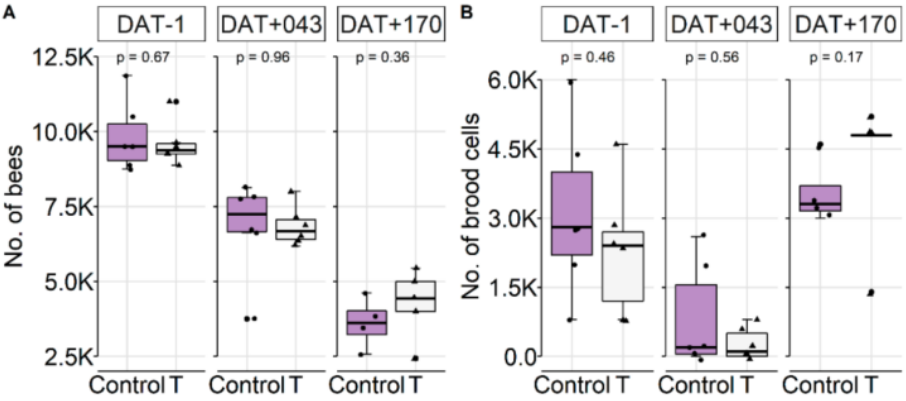

Figure 5. Effect of Roundup® Power Flex exposure (feeding) on colony conditions over winter, displayed as boxplot. (A) Number of worker bees. (B) Number of brood cells during the course of the study. For all assessments, conditions were not significantly different. Dates correspond to the following months: DAT−1 (1 October), DAT+43 (14 November), and DAT+170 (21 March).

Experiment 4: Determination of Glyphosate Based Herbicides Residues

In experiments 1, 2, and 3, irrespective of the time interval after the application (short term: three days, or long term: 170 days), glyphosate residues remained constant in all the sugar matrices.

In experiment 2, a 15.9-fold average increase, and a 24-fold peak increase was measured in stored pollen (beebread) when compared to stored nectar. In plants, a range similar to experiment 4 (tunnel tents) was measured shortly after the application on DAT0. Degradation of glyphosate in the tunnel tents, however, was faster than in the field.

In experiment 4, a matrix-dependent exposure gradient could be identified, which could be presented from high to low glyphosate residues, as follows: corbicular pollen > plants > honey sac > stored

food/nectar.
Honey sac residues were measured with a 3.3-fold reduction when compared to their floral source (plants). In turn, a 27.4-fold increase was measured in pollen when compared to honey sac contents, similarly to what was reported in experiment 2 for the stored products.
In experiment 1, trace residues of glyphosate were found in the pooled control food on DAT+21 (0.18 mg a.i./kg, AMPA was not detectable).
In experiment 3, trace residues of glyphosate were found in one control and one treatment colony before the application on DAT−1 (C2: 0.05 and T2: 0.04 mg a.i./kg, AMPA was not detectable).
In honey and plants, the LOD of GLY was 5.0 µg/kg and in pollen, it was 12.5 µg/kg, respectively. The LOQ was 12.5 µg/kg and 25 µg/kg, respectively. In honey, the LOD of AMPA was 2.5 µg/kg, and in plants and pollen, it was 12.5 µg/kg, respectively. The LOQ was 5.0 µg/kg and 25 µg/kg, respectively.

			Stock Solution		Stored Food/Nectar		Honey Sac	
	Time	Samples (n)	GLY	AMPA	GLY	AMPA	GLY	AMPA
Experiment 1: Brood development & survival	DAT0 (T1)	1	4.764 ± 0.424 +	0.020 ± 0.003 +	-	-	-	-
	DAT0 (T2)	1	137.566 ± 4.318 +	0.428 ± 0.001 +	-	-	-	-
	DAT+21 (T1)	5 *	-	-	5.103	0.013	-	-
	DAT+21 (T2)	4 *	-	-	99.861	0.319	-	-
Experiment 2: Field exposure	DAT0 (+0.5 h)	6 *	-	-	n.d.	n.d.	-	-
	DAT+1	6	-	-	0.327 ± 0.246	0.087 ± 0.194	-	-
	DAT+3	6	-	-	0.490 ± 0.455	n.d.	-	-
	DAT+6	6	-	-	0.347 ± 0.362	n.d.	-	-
	DAT+7	6	-	-	-	-	-	-
Experiment 3: Overwintering	DAT −1	12	-	-	0.004 ± 0.012	n.d.	-	-
	DAT+6	6	-	-	4.462 ± 1.651	0.006 ± 0.005	-	-
	DAT+13	6	-	-	5.159 ± 1.011	0.012 ± 0.004	-	-
	DAT+43	6	-	-	7.148 ± 3.234	0.033 ± 0.015	-	-
	DAT+170	6	-	-	4.985 ± 0.613	0.019 ± 0.004	-	-
Experiment 4: Residues under semi-field conditions	DAT0 (+1 h)	3 *	-	-	-	-	24.918	0.092
	DAT+1	3 *	-	-	-	-	-	-
	DAT+2	3 *	-	-	-	-	-	-
	DAT+3	3 *	-	-	-	-	-	-
	DAT+5	4	-	-	0.182 ± 0.225	n.d.	-	-

Figure 6. Glyphosate (GLY) and aminomethylphosphonic acid (AMPA) residues in stock solution, stored food/nectar and honey sack corresponding to the assessment date (DAT = day after treatment) and matrix from all four experiments (Treatment groups only). All values are rounded and presented as means (±SD) in mg a.i./kg where appropriate. *: pooled sample, +: analyzed twice, n.d.: not detectable, -: not measured).

			Beebread		Corbicular Pollen		Plants	
	Time	Samples (n)	GLY	AMPA	GLY	AMPA	GLY	AMPA
Experiment 1: Brood development & survival	DAT0 (T1)	1	-	-	-	-	-	-
	DAT0 (T2)	1	-	-	-	-	-	-
	DAT+21 (T1)	5 *	-	-	-	-	-	-
	DAT+21 (T2)	4 *	-	-	-	-	-	-
Experiment 2: Field exposure	DAT0 (+0.5 h)	6 *	-	-	-	-	96.429 ± 13.540 +	0.360 ± 0.005 +
	DAT+1	6	-	-	-	-	44.715 ± 1.429 +	0.255 ± 0.007 +
	DAT+3	6	-	-	-	-	33.634 ± 0.016 +	0.218 ± 0.017 +
	DAT+6	6	5.512 ± 6.922	0.007 ± 0.017	-	-	-	-
	DAT+7	6	-	-	-	-	31.051 ± 1.930 +	0.224 ± 0.007 +
Experiment 3: Overwintering	DAT −1	12	-	-	-	-	-	-
	DAT+6	6	-	-	-	-	-	-
	DAT+13	6	-	-	-	-	-	-
	DAT+43	6	-	-	-	-	-	-
	DAT+170	6	-	-	-	-	-	-
Experiment 4: Residues under semi-field conditions	DAT0 (+1 h)	3 *	-	-	614.841	2.122	82.407	0.217
	DAT+1	3 *	-	-	-	-	103.892	0.362
	DAT+2	3 *	-	-	-	-	62.845	0.229
	DAT+3	3 *	-	-	-	-	92.886	0.395
	DAT+5	4	-	-	-	-	-	-

Figure 7. Glyphosate (GLY) and aminomethylphosphonic acid (AMPA) residues in beebread, corbicular pollen and plants corresponding to the assessment date (DAT = day after treatment)

and matrix from all four experiments (Treatment groups only). All values are rounded and presented as means (±SD) in mg a.i./kg where appropriate. *: pooled sample, +: analyzed twice, n.d.: not detectable, -: not measured).

Conclusion

Glyfos Unkraut-Frei® showed no significant differences in bee survival between the treatments of 4.8 (T1) and 137.6 mg a.i./kg diet (T2) and the control. The hatching weight of adult worker bees was significantly lower (16.7% reduction) and the brood termination (BFD+21) significantly increased when treated with 137.6 mg a.i./kg diet, compared to the control.
Roundup® Power Flex did not significantly affect colony development either in summer or during overwintering.
The tested glyphosate based herbicides did not affect the lifespan of individuals, colony conditions, and overwintering, but delayed worker brood development when applied at a chronic high concentration.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this study three field and one semi-field tests were conducted in Germany to assess the effect of glyphosate based herbicides (Glyfos Unkraut-Frei® 360 g a.i./L and Roundup® Power Flex 480 g a.i./L) to honey bees (*A. mellifera*). The field studies assessed the effect on the brood and colony development, adult survival, and overwintering success of honey bees, while residues were measured, whereas the semi-field study determined residues of glyphosate based herbicides in different bee relevant matrices.
Glyfos Unkraut-Frei® showed no significant differences in bee survival between the treatments of 4.8 (T1) and 137.6 mg a.i./kg diet (T2) and the control, but hatching weight of adult worker bees was significantly lower (16.7% reduction) and the brood termination (BFD+21) significantly increased when treated with 137.6 mg a.i./kg diet, compared to the control. Roundup® Power Flex did not significantly affect colony development either in summer or during overwintering.
Therefore, the study shows that the tested glyphosate based herbicides did not affect the lifespan of individuals, colony conditions, and overwintering, but delayed worker brood development when applied at a chronic high concentration (137.6 mg a.i./kg diet).

This study has been classified as relevant (Category A acc. EFSA GD 2092, Point 5.4.1) and reliable without restrictions.

Assessment and conclusion by RMS: