Aan: 5.1.2.e Woo , Ctgb beleid

Van: 5.1.2.e Woo , 5.1.2.e Woo , 5.1.2.e Woo ; Ctgb ecotox

Datum: 19/10/2018

Betreft: Advisering artikelen effecten van glyfosaat op bijen naar aanleiding van een artikel in de Volkskrant waarover door De Groot (D66) kamervragen zijn gesteld

Referentie:
1. Motta, E. V. S., Raymann, K., and Moran N. A. 2018. Glyphosate perturbs the gut microbiota of honey bees. *PNAS*, 2018 115 (41) 10305-10310.
2. Dai P, Yan Z, Ma S, Yang Y, Wang Q, Hou C, Wu Y, Liu Y, Diao Q. 2018. The herbicide Glyphosate Negatively Affects Midgut Bacterial Communities and Survival of Honey Bee during Larvae Reared in Vitro. J Agric Food Chem, 2018 66 (29) 7786-7793.

Samenvatting:
De Groot (D66) heeft Kamervragen gesteld naar aanleiding van een artikel over negatieve effecten van glyfosaat op bijen. De kamervragen verwijzen naar een artikel in de Volkskrant. Dat is weer gebaseerd op een stuk in The Guardian. Naar Motta (2018) en Dai (2018) wordt in het Guardian-artikel gerefereerd over glyfosaat en bijendarmflora. Zij concluderen dat de darmflora van bijen verstoord kan worden door glyfosaat.

Discussie en conclusie Ctgb:
Het Ctgb is van mening dat de twee studies onvoldoende aantonen dat glyfosaat de darmflora dusdanig verstoort dat ingegrepen moet worden in de bestaande toelatingen.

De belangrijkste overwegingen hierbij zijn dat:

Exacte blootstelling niveaus van honingbijen niet betrouwbaar kon worden vastgesteld in Motta *et al.* 2018 en Dai *et al.* 2018 en geen dosis respons relatie laten zien;

De observaties van verstoring van de darmflora zijn waargenomen bij onrealistische hoge doseringen van glyphosaat, onder kunstmatige condities, in aanwezigheid van onvoldoende dieet, en met een hele kleine hoeveelheid bijen;

Soortgelijke studies zijn tot op heden nog niet meegenomen in de stofbeoordeling van glyfosaat. Zowel in het Renewal Assessment Report (RAR) van december 2013 en de bijbehorende evaluatie van peer reviewed literatuur zijn soortgelijke studies niet opgenomen.

Bij de herbeoordeling van de stof (expiratie datum 15/12/1922) zullen beide studies Dai 2018 en Motta 2018 worden meegenomen.

Beschrijving van en commentaar Ctgb op onderdelen van de studie:

General conclusion from the evaluation of public literature studies Motta *et al.* 2018[1] and Dai *et al.* 2018[2]

Based upon the evaluation of the two public literature studies, the following is concluded:

Both studies aimed to demonstrate that exposure of honeybees to glyphosate might impact the gut microbiome. Motta *et al.* also performed additional experiments aimed at determining whether that perturbation of the microbiome might lead to greater susceptibility to a pathogenic infection.

However, all of the experiments had major deficiencies that qualify them as unreliable and inconclusive. The main culprits were, for example, small sample sizes, unknown/unreported rearing conditions of bees and experimental conditions, and insufficient quality of diet. Specific deficiencies found for each study are listed in the study evaluations presented below. It was also impossible to foresee how the findings from the studies could potentially translate to more realistic situations in the environment (e.g. extrapolations from lab to field, realistic exposure, potential effects at colony level, etc.).

Taken together, the studies do not indicate that a re-evaluation of glyphosate registrations in the Netherlands is warranted.

[1] Motta, E. V. S., Raymann, K., and Moran N. A. Glyphosate perturbs the gut microbiota of honey bees. *PNAS*, 2018 115 (41) 10305-10310.

[2] Dai P, Yan Z, Ma S, Yang Y, Wang Q, Hou C, Wu Y, Liu Y, Diao Q. The herbicide Glyphosate Negatively Affects Midgut Bacterial Communities and Survival of Honey Bee during Larvae Reared in Vitro. *J Agric Food Chem,* 2018 66 (29) 7786-7793.

Motta *et al.* 2018. Glyphosate perturbs the gut microbiota of honey bees

Study abstract *(copied from the article): Glyphosate, the primary herbicide used globally for weed control, targets the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway found in plants and some microorganisms. Thus, glyphosate may affect bacterial symbionts of animals living near agricultural sites, including pollinators such as bees. The honey bee gut microbiota is dominated by eight bacterial species that promote weight gain and reduce pathogen susceptibility. The gene encoding EPSPS is present in almost all sequenced genomes of bee gut bacteria, indicating that they are potentially susceptible to glyphosate. We demonstrated that the relative and absolute abundances of dominant gut microbiota species are decreased in bees exposed to glyphosate at concentrations documented in the environment. Glyphosate exposure of young workers increased mortality of bees subsequently exposed to the opportunistic pathogen Serratia marcescens. Members of the bee gut microbiota varied in susceptibility to glyphosate, largely corresponding to whether they possessed an EPSPS of class I (sensitive to glyphosate) or class II (insensitive to glyphosate). This basis for differences in sensitivity was confirmed using in vitro experiments in which the EPSPS gene from bee gut bacteria was cloned into Escherichia coli. All strains of the core bee gut species, Snodgrassella alvi, encode a sensitive class I EPSPS, and reduction in S. alvi levels was a consistent experimental result. However, some S. alvi strains appear to possess an alternative mechanism of glyphosate resistance. Thus, exposure of bees to glyphosate can perturb their beneficial gut microbiota, potentially affecting bee health and their effectiveness as pollinators.*

Introduction

The effects of glyphosate exposure on size and composition of the honey bee gut microbiome was investigated in adults. The gut microbiome of honey bees is thought to consist of 8 core bacterial species: *Lactobacillus spp.* Firm-4, *Lactobacillus spp.* Firm-5 (phylum Firmicutes), *Bifidobacterium spp.* (phylum Actinobacteria), *Snodgrassella alvi*, *Gilliamella apicola*, *Frischella perrara*, *Bartonella apis*, and Alpha 2.1 (phylum Proteobacteria)[3]. Newly emerged workers are nearly free of gut bacteria and acquire their normal microbial community orally through social interactions with other workers during the first few days after emergence[4]. Bees deprived of their normal microbiota show reduced weight gain and altered metabolism, increased pathogen susceptibility, and increased mortality within hives[5].

Several experiments were performed in the study and the details of those studies were mainly presented in the Supplementary Information.

Results

Hive experiments

Two hive experiments were performed, in autumn and spring. In each experiment, the same procedure was followed, namely: 2000 adult bees were collected from a hive, split into 3 groups (control, 5 and 10 mg glyphosate/L), and placed into cup cages (40 bees per cup cage, totaling 16 cup cages per group). The bees were exposed to glyphosate during 5 days. Then 15 bees from each group were sampled (Day 0), and 600 bees from each group were returned to the hive (it is not reported how these were chosen, nor what was done with the other 1385 bees). At Day 3 post exposure (Day 3), 15 marked bees from each group were sampled from the hive. Fewer than 20% of returned bees were recovered from each

[3] Kwong WK, Moran NA (2016). Gut microbial communities of social bees. Nat Rev Microbiol 14:374–384.
[4] Powell JE, Martinson VG, Urban-Mead K, Moran NA (2014). Routes of acquisition of the gut microbiota of Apis mellifera. Appl Environ Microbiol 80:7378–7387.
[5] Motta *et al.* 2018 and references therein.

group at Day 3. Relative and absolute abundances of gut bacteria were assessed. The exposure levels chosen in the hive experiment (5 and 10 mg glyphosate/L, G-5 and G-10, respectively) are claimed to mimic those expected in fields, *i.e.* 1.4 – 7.6 mg glyphosate/L (a reference is made to another article). However, the Ctgb could not trace the values from the source literature, or the concentrations were reported for aquatic environments. It is not clear what type of correlation levels in water would have with the overall exposure of bees, particularly on a per-bee basis.

In the first hive experiment, at d0 glyphosate exposure had had little effect on the bee gut microbiome size (total bacteria number). The authors claim that the effects of glyphosate exposure on the bee gut microbiome were more prominent at day 3, after treated bees were returned to the hive. However, although the effects of G-5 treatment were statistically significant relative to the control, the abundance of bacteria in the control and the G-10 treatment were similar at d3. Therefore, the biological relevance of the results is not fully justified at d3. In the second hive experiment, significant differences were not found between the control and the treatments at either d0 or d3. The authors concluded that glyphosate did not negatively affect the bee gut microbiome size as a result of 5 days exposure to 5 and 10 mg glyphosate/L.

At d0, the relative and absolute abundances of the core species, *S. alvi*, were significantly lower in the G-10 group at d0, while not so at d3, which was unexplained. In the second hive experiment, a significant reduction in absolute abundance of *S. alvi* was observed at d0 and d3 in the G-10 treatment (although no clear concentration response could be established). The authors concluded that from of the eight "core" bacterial species in the honey bee midgut, only one species, *S. alvi,* shows indications of a potential decrease in total abundance as a result of 5 days of exposure to 10 mg glyphosate/L.

There are several shortcomings of the study, and the most critical ones are listed here: a) the sample size was 15 bees per treatment (2 sampling times = 30 bees), which is relatively low to provide an understanding of natural background variation versus actual effects, b) bees from only 2 hives were sampled (spring and autumn), c) the rearing conditions of bees were not reported, which might have influence on the results and is of importance to judge the health of bees, d) all bees were taken from and returned to the same hive – the test groups were therefore not isolated from each other and thus it is unknown whether a transfer of glyphosate and bacteria might have occurred among bees, e) variations in gut bacteria are common in different hives, but also between different individuals[6], but there was no way to compare this information in the experiments, nor any information provided by the study authors regarding this, f) the microbiome composition is influenced by age[7,8] and since the age of the bees in the experiment was not reported, and it is possible that bees of different ages were used, it is not clear whether it is appropriate to compare to other published information on the honey bee microbiome, or even, indeed, between the groups in the experiment, g) the exact amount of consumed sucrose syrup is not reported and the actual doses of glyphosate per bee per day therefore cannot be calculated, h) the bees were not fed with pollen (a source of proteins and enzymes for nurse bees), which may have influenced the results since many microbes are dependent upon amino acids for

6 Hroncova Z, Havlik J, Killer J, Doskocil I, Tyl J, Kamler M, et al. (2015) Variation in Honey Bee Gut Microbial Diversity Affected by Ontogenetic Stage, Age and Geographic Location. PLoS ONE 10(3): e0118707. https://doi.org/10.1371/journal.pone.0118707
7 Hroncova Z, Havlik J, Killer J, Doskocil I, Tyl J, Kamler M, et al. (2015) Variation in Honey Bee Gut Microbial Diversity Affected by Ontogenetic Stage, Age and Geographic Location. PLoS ONE 10(3): e0118707. https://doi.org/10.1371/journal.pone.0118707
8 Tarpy, D.R.; Matila, H.R. and Newton, I.L.G. (2015) Development of the Honey Bee Gut Microbiome throughout the Queen-Rearing process. *AEM* 81(9): 3182-3191.

survival, i) although the source of the glyphosate was reported, the GLP certification of the lab performing the test was not shown.

Taking into consideration the shortcomings listed above, the results of the hive experiment are not reliable and thus no conclusion can be drawn on glyphosate effect on gut microbiome size and absolute and relative abundances of the "core" species of the microbiome.

Colonization experiment

"Approximately 100" newly emerged workers (NEWs), which are supposedly nearly free of gut bacteria, were simultaneously exposed to an inoculum consisting of their normal microbial community and to glyphosate (5 µl of sucrose solution with 1 mM glyphosate ~1.7 µg glyphosate/bee; bees were exposed twice within 2 days). Of these, 15 bees were sampled in order to determine gut microbiome. In a second colonization experiment the exposure levels differed, *i.e.* the bees were exposed to 0.1 mM glyphosate during 5 days. Since no more information was available, it is not possible to calculate the total dose the bees consumed and to quantitatively compare the effects between the tests. In this case, only 8 bees per test group were sampled for DNA and RNA extraction.

In the first experiment, the average total bacterial abundance was slightly lower in glyphosate-treated bees, but this was not statistically significant. *S. alvi* was the most strongly affected member of the gut microbiota and its absolute and relative abundances were significantly lower in comparison with the control bees, while *Lactobacillus* Firm-4 increased in relative abundance only. The authors concluded that glyphosate exposure during early development of the gut community can interfere with normal colonization by altering the abundance of beneficial bacterial species.

In the second experiment, the authors also analyzed changes in bacterial abundance after glyphosate exposure by extracting both DNA and RNA from the guts of treated and control bees. A positive control group was included, in which bees were exposed to tylosin, an antibiotic used in beekeeping. This antibiotic treatment was expected to perturb the microbiota, but the decrease was significant only for RNA samples. Glyphosate exposure resulted in non-significant decreases in total bacteria for both DNA and RNA assays. Effects of glyphosate treatment on absolute abundance were specific to *S. alvi*, which was the only assayed species showing significant reductions in absolute abundance, observed for both DNA and RNA assays. The authors concluded that significant effects were observed only on absolute abundance of *S. alvi* DNA and RNA.

Most of the shortcomings mentioned in the hive experiments are applicable here in the colonization experiment, *e.g.* (even smaller) sample size, unknown doses, lack of accounting for natural variations in gut microbiome among individuals and so forth. As a result, the colonization experiments are also considered unreliable and inconclusive.

Infection experiments

To determine whether glyphosate-induced perturbation of microbiota colonization affects host health, the susceptibility of glyphosate-treated bees to an opportunistic bacterial pathogen was measured in two experiments. NEWs were exposed to glyphosate during the stage of acquiring their normal microbial community. After 5d of treatment (first experiment: 0.1 mM glyphosate over 5 days; second experiment: 0.1 mM glyphosate over 5 d ~1.7 µg glyphosate/bee (assuming bees take on average 20 µl sucrose solution per day, the same likely applies for the first experiment, but it was not reported)), bees were challenged with *Serratia marcescens* kz19, an opportunistic pathogen commonly detected at very low frequencies in the bee gut. For bees lacking gut microbiota, *Serratia* challenge resulted in increased

mortality relative to that observed for bees with a conventional gut microbiota, regardless of glyphosate exposure. For bees with a conventional gut microbiota, glyphosate treatment resulted in increased mortality after *Serratia* challenge. In bees exposed to glyphosate, but not challenged with *Serratia*, survival rates were not significantly affected by glyphosate and much higher (they were actually the highest from all tested groups) than in the *Serratia*-challenged groups, demonstrating that a direct effect of glyphosate on bees is not the basis of the high mortality of glyphosate-exposed, pathogen-challenged bees.

It was suggested by the authors, based upon the results above, that glyphosate reduces the protective effect of the gut microbiota against opportunistic pathogens and that *S. alvi* is the bacterial species most negatively affected by glyphosate exposure. The authors pointed out that *S. alvi* appears to give some immune protection, but not as fully as the whole gut microbiota.

The authors further hypothesize the reasons for the observed variation in sensitivity of certain strains of bacterial species toward glyphosate, which is outside of the scope of the present evaluation for ecological risk assessment. Only short conclusion on the topic is presented here: It was concluded that bee gut bacteria vary in glyphosate sensitivity at the species and strain levels and that *S. alvi* strains may vary in sensitivity to glyphosate in vivo. Thus, strain differences in glyphosate sensitivity may potentially contribute to the observed variation in the overall decrease in *S. alvi* abundance when bees with their native gut microbiota are exposed to glyphosate.

It must be highlighted that it is not clear how the dose of the injected pathogen relates to potentially realistic doses (*i.e.* to what levels of *Serratia* the bees are naturally exposed). The authors state that the pathogen is naturally present in the gut at low frequencies, so injecting the pathogen does not seem to reflect natural situation. It is also not clear whether the possible observed effects were a consequence of the infection itself, or a consequence of infection and exposure together to glyphosate (as bees have a higher energy demand when exposed to two stressors), or a consequence of alterations of the honey bee immune response as a result of unaccounted for factors. Furthermore, the authors mention that the guts from 10 bees were pulled out, prepared, and were given to bees during 5 days until normal microflora was established. However, it is questionable what is the "normal" microflora, *i.e.* what is the baseline composition of the microflora. In addition, the bees were not fed with pollen (a source of proteins and important enzymes from nurse bees), which may influence the results as discussed above. Lastly, the doses were inferred by assuming that bees it 20 µl sucrose syrup per day, while in the EFSA GD on Bees (2013), the amount of the sugar bees consume is higher (32-128 and 34-50 mg/bee/day for foragers and nurses, respectively), and therefore the exact exposure dose reported is considered incorrect or at least uncertain. Overall, the colonization experiments are considered not sufficiently reliable and the results are inconclusive.

Conclusion

Several experiments were conducted in the study to show that exposure of adult bees might impact the gut microbiome leading to a greater susceptibility to pathogen infections.

However, based on a number of shortcomings in each experiment (*e.g.* very small sample sizes, unknown rearing and experimental conditions, influence of hive, individual and age on gut microbiome, lack of confirmation of the levels of actual glyphosate exposure per bee, bees diets had no source of amino acids, injection of a pathogen at unjustified levels, etc.), the study was considered unreliable and

inconclusive. Furthermore, the study does not reflect environmentally realistic settings, nor are other potential variables affecting the gut microbiome excluded.

It is interesting to note that although the authors intimate that due to the posited effects on the microbiome glyphosate may have a roll in colony collapse, they themselves use, as a positive control for gut microbiome perturbation, an antibiotic commonly used in bee-keeping.

Taken together, the study does not indicate that a re-evaluation of glyphosate registrations in the Netherlands is warranted.

Dai et al. 2018. The herbicide glyphosate negatively affects midgut bacterial communities and survival of honey bee during larvae reared in vitro

Study abstract *(copied from the article): Effects of glyphosate on survival, developmental rate, larval weight, and midgut bacterial diversity of Apis mellifera were tested in the laboratory. Larvae were reared in vitro and fed diet containing glyphosate 0.8, 4, and 20 mg/L. The dependent variables were compared with negative control and positive control (dimethoate 45 mg/L). Brood survival decreased in 4 or 20 mg/L glyphosate treatments but not in 0.8 mg/L, and larval weight decreased in 0.8 or 4 mg/L glyphosate treatments. Exposure to three concentrations did not affect the developmental rate. Furthermore, the intestinal bacterial communities were determined using high-throughput sequencing targeting the V3−V4 regions of the 16S rDNA. All core honey bee intestinal bacterial phyla such as Proteobacteria (30.86%), Firmicutes (13.82%), and Actinobacteria (11.88%) were detected, and significant changes were found in the species diversity and richness in 20 mg/L glyphosate group. Our results suggest that high concentrations of glyphosate are deleterious to immature bees.*

Introduction

The study aimed to develop an approach to evaluate potential effects of glyphosate on honey bee brood reared in vitro. The authors evaluated survival, developmental rate, larval weight, and midgut bacterial communities of in vitro-reared honey bees exposed chronically to varying concentrations of glyphosate as larvae under controlled laboratory conditions. According to the authors, the concentrations of glyphosate (0.8, 4, and 20 mg glyphosate/L, G-0.8, G-4, G20, respectively) were based on concentrations recommended for spraying and on those measured in natural environments, from 1.4 to 7.6 mg/L. The authors stated that 20 mg/L glyphosate is unlikely to be encountered in the field and thus represents a worst case scenario. Regardless, for the "environmentally relevant" concentrations, either the Ctgb could not trace the values from the source literature, or the concentrations were reported for aquatic environments, making their relevance for honey bee exposure uncertain.

The study design resembles the OECD Series on Testing and Assessment No. 239 "Larval toxicity test with repeated exposure". The bee larvae were exposed to repeated doses of glyphosate on days 2 to 5. On day 6, the larvae were transferred to chambers for pupation. Once emerged, the young bees were sampled for the screening of intestinal gut microflora. A GLP certificate was not available.

Results

By day 18, significant effects were observed on survival in G-4 and G-20 treatments. Although the values were not reported (only the graphical representation was given), survival of ~85 and ~75% can be inferred from the figure for G-4 and G20, respectively. Nevertheless, according to the EFSA GD on Bees (2013), the observations should be made on day 7 of the test, for which the survival was approximately 90% or above in G-4 and G-20. Furthermore, although the effects were statistically significant, they can be considered a result of natural variation – one of the validity criteria for the test (OECD Series on Testing and Assessment No. 239) is a minimum 70% emergence (*i.e.* survival) by day 22. Since the survival in all treatments was above 70%, the relationship between glyphosate exposure and effects cannot be established.

The authors stated that the effects on larval fresh weight were observed on day 6 in G-0.8 and G-4, but not in G-20. Nevertheless, the Ctgb is of the opinion that the differences are very minor and that no dose response was established. Therefore, the "effects" on larval weight can also be considered *e.g.* a result of natural variation.

Larval and pupal developmental rates were not affected.

By using Hierarchical cluster analysis, differences in the midgut microbial community composition was demonstrated in the G-20 treatment group. However, interestingly, compared to the work of Motta *et al.* 2018, no significant difference in Betaproteobacteria (the class of bacteria to which *S. alvi* belongs) was obvious from this analysis. Furthermore, regarding the specific taxa, although some differences were observed in each treatment, no consistent alterations of particular taxa were observed – for example, the abundance of Gemmatimonadaceae was significantly higher in G-0.8 treatment, but not in G-4 and G20, and so forth. Moreover, the results were obtained only from five individuals per treatment. Therefore, the results are considered inconsistent and unreliable.

Overall, the authors state that their findings confirm the previous work on glyphosate that found no effects on brood development in a realistic exposure scenario. In a recent study[9], peak glyphosate residues after application at 2.88 kg/ha were 31.3 mg/kg in nectar and 574 mg/kg in pollen at the first 3 days of exposure, and glyphosate demonstrated rapid decline to 2.78 mg/kg in nectar and 87.2 mg/kg in pollen by day 7. The authors in Dai et al. 2018 state that it seems unlikely that the glyphosate levels found in brood food will approach the maximum residues found in pollen or nectar/honey under normal environmental conditions. In addition, the authors suggest that their study was conducted under artificial circumstances, and that in a more realistic situation larvae and young bees would have opportunities for a transfer of microflora between their counterparts, and would attain their gut bacteria from older workers, the hive environment, or a combination of the two. This would "dissolve" any potential effect of the composition of gut microflora.

Conclusion

No effects could be detected on survival, fresh weight, and developmental rates of larvae and pupae, even at concentrations that may considered unrealistically high in the environment. No consistent effects were observed on gut microflora numbers and composition. Moreover, the findings are not consistent with the findings in the study of Motta *et al.* 2018 regarding the bacteria species *S. alivi.* And finally, the study on the gut microflora was performed with only five individuals per treatment, which is considered insufficient to account for natural variation. Overall, the study is considered unreliable and inconclusive.

Taken together, the study does not indicate that a re-evaluation of glyphosate registrations in the Netherlands is warranted.

[9] Thompson, H.M., Levine, S.L., Doering, J., Norman, S., Manson, P., Sutton, P., von Mérey, G. Evaluating exposure and potential effects on honeybee brood (Apis mellifera) development using glyphosate as an example. *Integr Environ Assess Manag* 2014 10 (3) 463-70.