FEMS Microbiology Ecology, 2023, **99**, 1–10
DOI: 10.1093/femsec/fiad065
Advance access publication date: 14 June 2023
Research Article

Glyphosate and a glyphosate-based herbicide affect bumblebee gut microbiota

Marjo Helander[1,*], Aditya Jeevannavar[1], Kimmo Kaakinen[1], Suni A. Mathew[1], Kari Saikkonen[2], Benjamin Fuchs[2], Pere Puigbò[3,4], Olli J. Loukola[5], Manu Tamminen[1]

[1]Department of Biology, University of Turku, 20014 Turku, Finland
[2]Biodiversity Unit, University of Turku, 20014 Turku, Finland
[3]Nutrition and Health Unit, Eurecat Technology Centre of Catalonia, 43204 Reus, Catalonia, Spain
[4]Department of Biochemistry and Biotechnology, Rovira i Virgili University, 43002 Tarragona, Catalonia, Spain
[5]Ecology and Genetics Research Unit, University of Oulu, 90014 Oulu, Finland
*Corresponding author. Department of Biology, University of Turku, 20014 Turku, Finland. E-mail: helander@utu.fi
Editor: [Lise BARTHELMEBS]

Abstract

Pollinator decline is one of the gravest challenges facing the world today, and the overuse of pesticides may be among its causes. Here, we studied whether glyphosate, the world's most widely used pesticide, affects the bumblebee gut microbiota. We exposed the bumblebee diet to glyphosate and a glyphosate-based herbicide and quantified the microbiota community shifts using 16S rRNA gene sequencing. Furthermore, we estimated the potential sensitivity of bee gut microbes to glyphosate based on previously reported presence of target enzyme. Glyphosate increased, whereas the glyphosate-based herbicide decreased gut microbiota diversity, indicating that negative effects are attributable to co-formulants. Both glyphosate and the glyphosate-based herbicide treatments significantly decreased the relative abundance of potentially glyphosate-sensitive bacterial species *Snodgrasella alvi*. However, the relative abundance of potentially glyphosate-sensitive *Candidatus Schmidhempelia* genera increased in bumblebees treated with glyphosate. Overall, 50% of the bacterial genera detected in the bee gut microbiota were classified as potentially resistant to glyphosate, while 36% were classified as sensitive. Healthy core microbiota have been shown to protect bees from parasite infections, change metabolism, and decrease mortality. Thus, the heavy use of glyphosate-based herbicides may have implications on bees and ecosystems.

Keywords: bumblebees, gut microbiota, herbicides, pesticides, pollination, resistance

Introduction

By providing essential pollination services, pollinators are key players in maintaining global biodiversity, ecosystem functioning, and agricultural productivity (Potts et al. 2010, 2016, Reilly et al. 2020). Growing evidence points to substantial losses of pollinators in many regions of the globe. Both the abundance and diversity of insect pollinators have alarmingly declined in Europe and Northern America, as well as in other parts of the world during the last decades (Ghazoul 2005, Potts et al. 2010, Hallman et al. 2017, Reilly et al. 2020, Zattara and Aizen 2021). These declines are observed in both the wild insect populations such as bumblebees (*Bombus* spp.) and the domesticated honey bee (*Apis mellifera*) stocks, which are essential in horticulture and agriculture, and provide pollination service to wild plant communities. For example, in Germany, the biomass of flying insects has declined by 75% in the previous quarter century (Hallman et al. 2017), and the number of bee species has dropped by ∼25% globally since the 1980s (Zattara and Aizen 2021).

Land cover, configuration, management, and pesticide use are indisputably the most important culprits responsible for pollinator decline worldwide (Goulson et al. 2015, Carvell et al. 2017, Dicks et al. 2021, Helander et al. 2023). Other important drivers of this decline include pests and pathogens, the use of genetically modified (GM) crops, invasive alien species, and climate change (Dicks et al. 2021). However, the impacts of individual drivers are often difficult to separate from those of other drivers. For example, the world's most used pesticides, glyphosate-based herbicides, are commonly applied to control weeds in agriculture, horticulture, silviculture, and urban environments. Furthermore, since the development and launch of the first widely used GM crop 'Roundup Ready' soybean, a GM crop resistant to glyphosate, in the market in 1996, the farming of genetically engineered crops resistant to herbicides has massively increased (Benbrook 2016). This has led to greater use of glyphosate-based herbicides. Since the expiry of the glyphosate patent in 2000, glyphosate-based herbicides have become the most used pesticides globally (Benbrook 2016). Further, climate change has facilitated species range expansions and invasions into new environments (Chen et al. 2011), and the size of farms has increased in many areas, including North America, Argentina, Australia, and many European countries (Lowder et al. 2016), further expanding the need for crop protection, as large monocultures are exceptionally vulnerable to weeds, pests, and pathogens. Distinguishing the impact of individual drivers acting simultaneously, therefore, requires experimental research.

Glyphosate has been assumed to be safe for humans and animals because it targets a key enzyme of the shikimate pathway, 5-enolpyruvylskhikimate-3-phosphte synthase (EPSPS), which synthesizes essential amino acids in plants. However, an increasing

Received 6 April 2023; **revised** 26 May 2023; **accepted** 13 June 2023

number of studies have demonstrated risks to animals as well, because the shikimate metabolic pathway is present in many microbes affecting their health outcomes (Leino et al. 2021, Rainio et al. 2021, Ruuskanen et al. 2023). In addition to the active ingredient glyphosate, glyphosate-based herbicides include co-formulants, which can be even more toxic to nontarget organisms than glyphosate alone (Defarge et al. 2018, Helander et al. 2019, Straw et al. 2021). However, often, only the active ingredients are required to be tested for their toxicity to nontarget organisms (Mesnage et al. 2019). Thus, to fully understand how glyphosate-based herbicides may affect pollinators, we need better knowledge of how glyphosate and the other ingredients of glyphosate-based herbicides separately modulate microbiomes such, as gut microbiomes, which are essential to pollinator wellbeing.

Increasing evidence demonstrates that glyphosate-based herbicides modulate the diversity and community composition of animal microbiomes (Ruuskanen et al. 2020, 2023, Leino et al. 2021) and that gut microbiota play a significant role in the health of their animal hosts (Koch and Schmid-Hempel 2011, Lee and Hase 2014, Rooks and Garrett 2016). Recent studies indicate that the gut microbiota may influence the brain functions and behaviour of animals (Cryan and Dinan 2012, Lee and Hase 2014, Teseo et al. 2019, Li et al. 2021). Glyphosate affects the microbiota of honeybees by reducing the abundance of beneficial bacterial species that contribute to immune regulation and pathogen resistance and this disruption of the normal microbiota leads to increased mortality (Motta and Moran 2020). Cullen et al. (2023) investigated the impact of chronic exposure to field-relevant doses of glyphosate and RoundUp Optima+® on bumblebees and found alterations in digestive tract proteome and microbiota without any impact on survival, behaviour, or food consumption. Tang et al. (2023) found that sublethal exposure to glyphosate significantly increased the activity of prophenoloxidase and altered the structure of the dominant gut fungal community, reducing the relative abundance of Zygosaccharomyces associated with fat accumulation in bumblebees.

Here, we investigate the effects of the world's most used pesticide, glyphosate, on bumblebee (*Bombus terrestris*) microbiota. Bumblebees are effective native pollinators of wild flowering plants and economically important crops, and very little is known about the effects of glyphosate or glyphosate-based herbicides on bumblebee gut microbiota (Motta and Moran 2023). We exposed bumblebee colonies to chronic low and high pure glyphosate or glyphosate-based herbicide treatment for 3 or 5 days and investigated their gut microbiota communities. We determined the bumblebee gut microbiota community composition and community shifts in response to the treatments using 16S rRNA gene sequencing. The sensitivity of the community members to glyphosate was estimated computationally based on the previously reported presence of the EPSPS enzyme in the detected bacterial genera (Leino et al. 2021). These results were compared with the core microbes detected in our study to investigate whether glyphosate-sensitive and glyphosate-resistant microbes respond differently when they are exposed to glyphosate or glyphosate-based herbicides.

Materials and methods

Chronic exposure of bumblebee colonies to glyphosate-based herbicide and glyphosate

Bumblebees (*B. terrestris*) from Koppert, The Netherlands, were used in the experiments. Bees deposit their crop load into the hive between each foraging trip, and therefore expose other colony members to glyphosate-based herbicides and glyphosate. All members in a *B. terrestris* colony are full sisters, because the founding queen mates with one male (Schmid-Hempel and Schmid-Hempel 2000), thus limiting the genetic variation within the hive. Furthermore, the gut microbiota of newly emerging bumblebee workers is inoculated by the founder queen and other older workers (Hammer et al. 2021), leading to the similarity of microbiota within the colony members. Therefore, comparing the gut microbiota of control bumblebees (bees from the hive before they are exposed to treatments) with gut microbiota from glyphosate or glyphosate-based herbicide-treated bumblebees allows us to estimate the effects of the treatments on the known microbial community.

All the exposure treatments were conducted in the summer of 2022 at Turku University Bumblebee Laboratory indoors in 10/14 h light–dark cycle and temperature between 22°C and 23°C. The bumblebees were housed in a two-chamber wooden nesting box connected to a flight arena (60 × 45 × 25 cm, with a transparent top) by an acrylic tunnel. Bumblebees were allowed to move freely between the hive and the flight arena, where they could feed on 60% sucrose solution *ad libitum* for 2 days, after which entering the arena was prevented by placing a sling door to the tunnel. From each colony, five actively foraging control bumblebees in the foraging areana (total 30 bees) (Day 0 = control) were removed, placed into an Eppendorf-tube, immediately snap-frozen in liquid nitrogen, and stored in a deep-freezer at −80°C. Thereafter, we exposed the hive either to pure glyphosate (Sigma Aldrich, Pestanal, $(HO)_2P(O)CH_2NHCH_2CO_2H$, molecular weight 169,07 g mol^{-1}) in 60% sucrose solution or a glyphosate-based herbicide Roundup (commercial product Roundup Gold, Monsanto Europe S.A., Belgium, registration number 1934; glyphosate concentration 450 g L^{-1}, as glyphosate isopropylamine salt CAS: 3 864 194–0). The professional formulation was purchased from an agricultural retailer Hankkija, Finland (www.hankkija.fi). Two bumblebee colonies were exposed to a low concentration of 10 mg L^{-1} of pure glyphosate, one colony to a high concentration of 5 g L^{-1} of pure glyphosate, two colonies to field realistic (Motta et al. 2018) Roundup concentration of 10 mg L^{-1} of active ingredient glyphosate, and one colony was exposed to a high Roundup concentration of 5 g L^{-1} of active ingredient glyphosate in 60% sucrose solution. The 10 mg L^{-1} of glyphosate concentration was chosen, because comparable concentrations have been used in studies with honey bees (Dai et al. 2018, Motta et al. 2018, Blot et al. 2019, Motta and Moran 2020, 2020, Castelli et al. 2021, 2022). The recommended dilution for glyphosate-based herbicide spray in the field is 3%–6% in water equaling glyphosate concentration of 13.5–27 g L^{-1}. Once sprayed on flowering plants, glyphosate-based herbicide will ultimately mix with the nectar of the flowers. After the bumblebees of each colony had been feeding *ad libitum* on either pure glyphosate or Roundup sugar water for 3 (Day 3) or 5 days (Day 5), five randomly selected actively foraging bumblebee workers in the foraging arena were removed from each colony, placed in an Eppendorf-tube, flash frozen in liquid nitrogen, and stored at −80°C. Together, we had five control, five 3-day exposed and five 5-day exposed bees from each of the six colonies.

From each of the experimental hives, one extra 10 mg L^{-1} Roundup hive, and three hives that were not used in the experiment (controls for the experimental hives), we marked 30 individual forager bees by super-gluing a small plastic number tag (Bienen-Voigt & Warnholz, Germany) on their thorax. We removed and counted all dead marked bumblebee individuals from each hive during 7 days after the treatments started.

Bumblebee gut microbiota analyses

Individual bees were rinsed in 70% ethanol, and their intestines, including the crop, midgut, and hindgut, were aseptically removed and immediately placed on Eppendorf-tubes on ice. Total genomic DNA was extracted using ZymoBiomics DNA Microprep kits (Zymo Research) according to the manufacturer's standard protocol. DNA libraries were prepared following the Earth Microbiome Project's protocol for 16-S-Illumina amplicons with minor modifications (Caporaso et al. 2011, 2012; www.earthmicrobiome.org/protocols-and-standards/16s/). The V4 region of the 16S rRNA gene was amplified using 515FB (5′GTGYCAGCMGCCGCGGTAA3′) and 806RB (5′GGACTACNVGGGTWTCTAAT3′) tailed primers that added Illumina adapters and unique barcodes to the amplicon. Negative controls from DNA extraction and PCR setup were added. A mock community sample of known species composition was also included (ZymoBIOMICS Microbial Community DNA Standard). The amplification reaction consisted of 1X Platinum™ II Hot-Start PCR Master Mix, forward and reverse primers, each at a final concentration of 0.2 μM, and 1 μL template DNA and PCR-grade water, in a total reaction volume of 10 μL. The PCR profile included an initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 15 s, and extension at 68°C for 15 s. Individual libraries were quantified with the DNA HS Assay on a Qubit fluorometer and pooled equimolarly, after which the pooled library was SPRI bead purified. Sequencing was performed by the Finnish Functional Genomics Centre (Turku, Finland) on Illumina MiSeq using 2 × 250 bp paired-end reads. Sequences were grouped into amplicon sequence variants (ASVs), and their phylogenetic affiliation was determined using a naïve Bayesian classifier implemented in DADA2 (Callahan et al. 2016). These analyses were carried out using the Ampliseq pipeline version 2.4.0 (Straub et al. 2020), and all bioinformatics analyses were performed on the Puhti supercomputing cluster (Atos BullSequana X400) provided by the Center for Scientific Computing (CSC Oy, Espoo, Finland). Alpha and beta diversity measures were determined using functions implemented in mia (Ernst et al. 2022) and vegan (Oksanen et al. 2022) packages, respectively. The significance of the community shifts was tested using a permutational ANOVA implemented in the vegan package. Genus-level phylogeny was inferred using functions implemented in mia. Significantly responding ASVs were determined using ALDEx2 (Fernandes et al. 2013), ANCOM-BC (Lin et al. 2022), MaAsLin2 (Mallick et al. 2021), LinDA (Zhou et al. 2021), and DESeq2 (Love et al. 2014). These analyses were run on R version 4.2.

Susceptibility of bumblebee gut microbes to glyphosate: a bioinformatics approach

The potential susceptibility or resistance of the detected bumblebee gut microbes to glyphosate was determined computationally based on the type of EPSPS enzyme reportedly present in microbial genera (Leino et al. 2021, Mathew et al. 2022). The EPSPS enzyme is the direct target of glyphosate in plants and microbes. EPSPS can be classified as potentially sensitive (class I) or resistant (class II–IV) to glyphosate based on amino acid markers present at its active site (Leino et al. 2021). The intrinsic sensitivity of bacteria to glyphosate was determined using amino acid markers in the EPSPS protein sequence using the EPSPSClass web-server (Leino et al. 2021). In cases where the exact bacterial species was unknown, estimates of potential sensitivity to glyphosate were obtained from Rainio et al. (2021) or estimated following the protocol described by Mathew et al. (2022).

Results

Bumblebee gut microbiota diversity is affected by glyphosate and roundup exposures

In the end of the experiment (on Day 7 from the beginning) the cumulative mortality of the control bees was 15.7%, lower Roundup treatment (10 mg L^{-1}) 15.0%, high glyphosate treatment (5 g L^{-1}) 34.4%, and higher Roundup treatment (5 g L^{-1}) 65.6% (Supplementary Fig. S1). Thus, the lower Roundup treatment (10 mg L^{-1}) can be considered as sublethal exposure, while the high Roundup treatment clearly increased bumblebee mortality.

Sequencing of the 16S rRNA genes resulted in ∼8 million reads, which were grouped into 128 ASVs belonging to 47 genera. On average, 77 807.45 reads were obtained per sample, with sequencing reaching a plateau (Supplementary Fig. S2). The Shannon index was calculated as an alpha-diversity measure and compared between the glyphosate and Roundup exposures (Fig. 1A and B, respectively) and over the observation period of 5 days for glyphosate and Roundup treatments (Fig. 1C and D, respectively). The community diversity significantly increased for the glyphosate treatment and significantly decreased for the Roundup treatment at the lower (10 mg L^{-1}) glyphosate exposure (Fig. 1A and B for glyphosate and Roundup treatments, respectively; Wilcoxon test; $P < .01$). The community diversity significantly increased in the glyphosate treatment over the observation period of 5 days (Fig. 1C; Wilcoxon test; $P < .05$) was not significantly affected by Roundup treatment (Fig. 1D; Wilcoxon test; $P < .05$).

Bumblebee gut microbiota composition is affected by glyphosate and Roundup exposures

All microbial gut communities were dominated by phyla *Proteobacteria* and *Firmicutes*, and no community responses could be identified on this phylogenetic level (Supplementary Fig. S3A). The most common genera detected in gut microbiota at the beginning of the experiment included *Snodgrasella* (38.46% ± 20.82%) and *Gilliamella* (39.85% ± 23.69%) whereas glyphosate and Roundup treatments shifted the community towards a higher relative abundance of *Candidatus Schmidhempelia* (18.46% ± 20.90%), *Klebsiella* (26.81% ± 35.66%), and *Lactobacillus* (11.14% ± 15.36%) (Supplementary Fig. S3). To test whether the glyphosate and Roundup exposures affected the bumblebee gut microbiota composition, a redundancy analysis was performed using the ASVs as the response variables and the *treatment*, *dose*, and *hive* origin of the bumblebees as the explanatory variables. The microbiota was significantly affected by these explanatory variables (permutational ANOVA $P < .01$), with the first two axes explaining 33.1% of the bacterial variation (Fig. 2). The most affected ASVs were the genera *Klebsiella*, *Gilliamella*, and *Snodgrasella* (Table 1).

Sensitivity of bee gut microbes to glyphosate and Roundup

The genera affected by exposure type (glyphosate or Roundup), exposure dose (10 mg L^{-1} or 5 g L^{-1}) and exposure duration (control = Day 0, Day 3, or Day 5) were identified using a combination of five differential abundance estimators: ALDEx2 (Fernandes et al. 2013), ANCOM-BC (Lin et al. 2022), MaAsLin2 (Mallick et al. 2021), LinDA (Zhou et al. 2021), and DESeq2 (Love et al. 2014). The estimators were queried at a significance level of $P < .05$ after Benjamini–Hochberg-correction, and responses in which more than three of the five estimators indicated a significant response were considered significant. The significantly affected

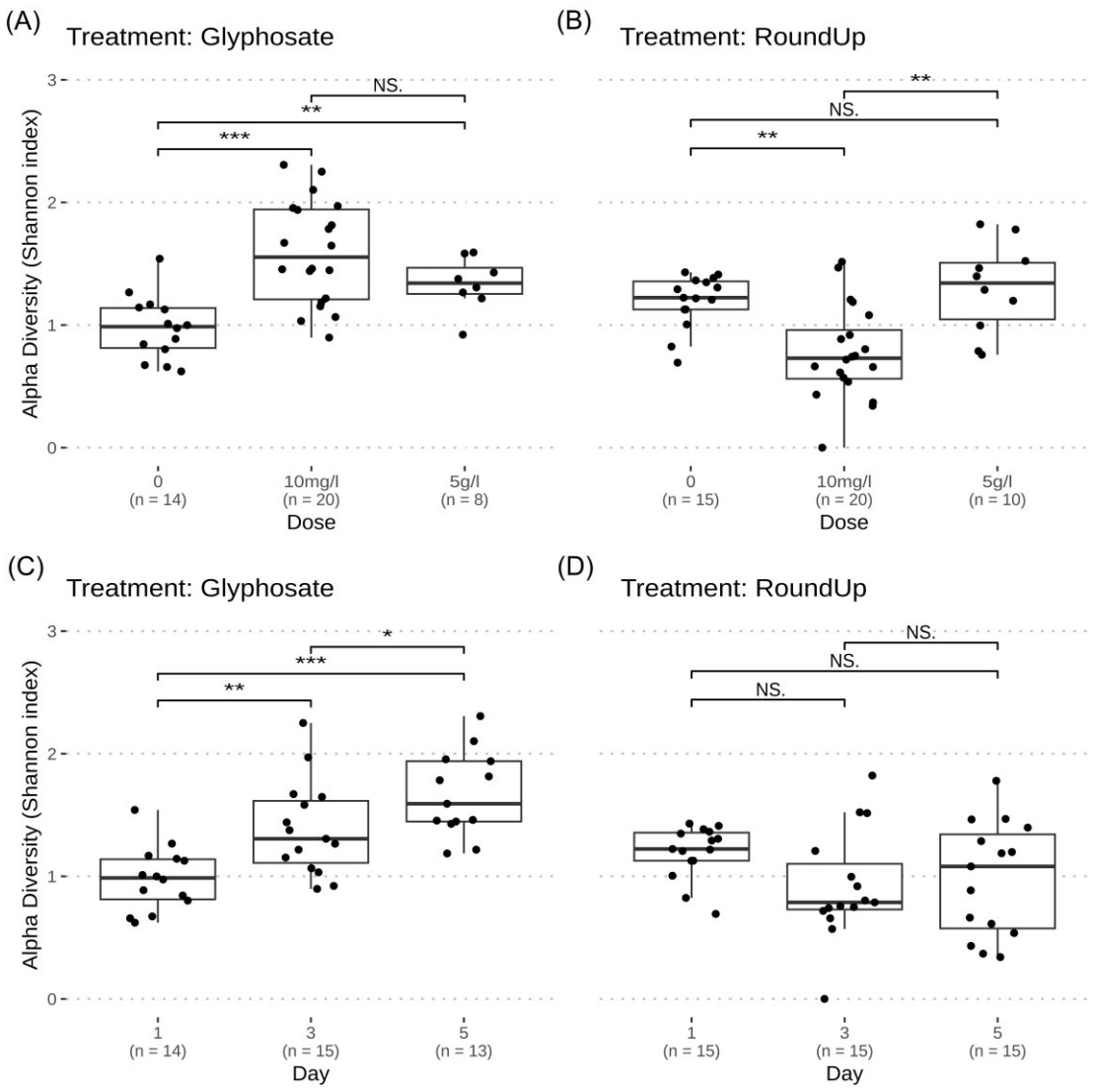

Figure 1. Boxplots of the Shannon diversity indexes indicate significantly higher and significantly lower diversities for (A) glyphosate and (B) Roundup (10 mg L^{-1}), respectively, when compared to the control (Wilcoxon test; ***P-value < .001, **P-value < .01, *P-value < .05). (C) Glyphosate treatment increases community diversity over the observation period of 5 days (Wilcoxon test). (D) Roundup treatment slightly decreases community diversity on Day 3 of the observation period (Wilcoxon test). The *n* numbers below the boxes indicate the number of samples used for the comparisons.

genera are indicated in their phylogenetic context in Fig. 3A and indicate, among the large-scale effects, increased abundance of *Klebsiella* and *Bacillus* and decreased abundance of *Snodgrasella* ASVs in response to glyphosate and Roundup treatments. All significant comparisons are indicated in Fig. 3B, and also include genera such as *Acinetobacter*, *Candidatus Schmidhempelia*, and *Weissella* with increased abundance in response to glyphosate, and *Klebsiella* and *Snodgrasella* with increased and decreased abundance in response to Roundup, respectively.

Predicting gut microbe sensitivity to glyphosate

Of the bacterial ASVs in the microbiota 50.0% were classified as resistant, 36.1% were classified as sensitive, and 13.9% remained unclassified in their potential sensitivity to glyphosate (EPSPSClass web-server, Leino et al. 2021; Supplementary Table S1). This result is in line with the potential bacterial sensitivity to glyphosate found in bioinformatics analyses of human microbiota (Leino et al. 2021, Puigbò et al. 2022), but should be taken as a conservative estimate, which does not consider short-term changes in the EPSPS sequence due to glyphosate exposure. The analysis of ASV counts from our experiment indicates a clear dominance of glyphosate-sensitive bacteria representing >80% of ASVs in the dataset (Fig. 4).

Discussion

Here, we demonstrated that a field-realistic exposure (10 mg L^{-1}) of either pure glyphosate or glyphosate in glyphosate-based herbicide (Roundup) altered the bumblebee gut microbiota. However, the effects were inconsistent between the glyphosate and Roundup treatments; pure glyphosate increased but Roundup decreased gut microbiota diversity. Both exposures (10 mg L^{-1} and 5 g L^{-1}) of pure glyphosate treatment increased the community composition of the gut microbiota. In contrast, the community diversity response to the Roundup was concentration-dependent. The low exposure of the Roundup treatment decreased gut microbiota diversity, whereas the high exposure did not lead to decreased community diversity in the treated bees compared to

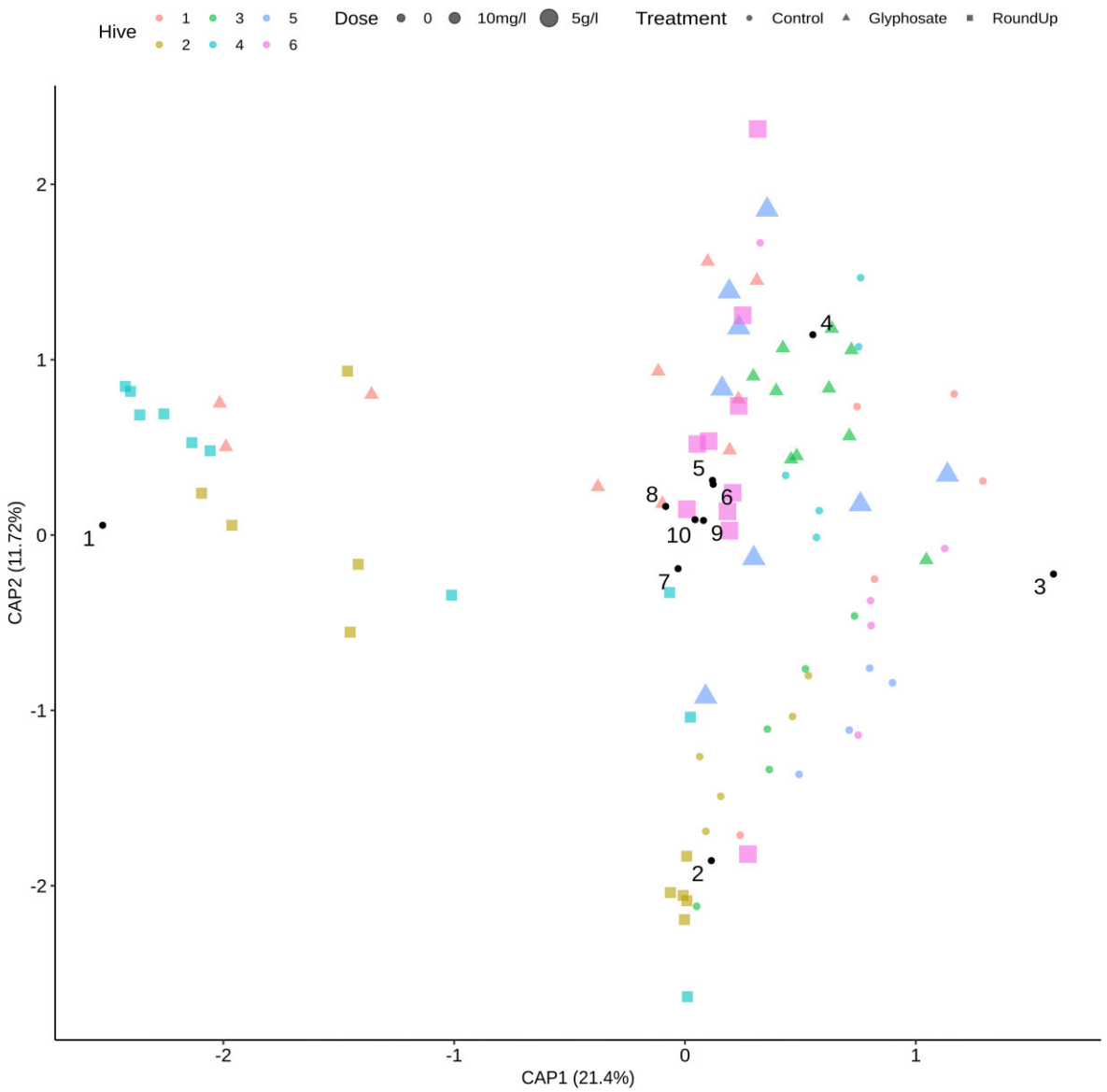

Figure 2. Samples and ten most variable ASVs plotted on the first two main axes from redundancy analysis of the bacterial community response to glyphosate and Roundup treatment, treatment dose, and hive. The community response is significant (permutational ANOVA $P < .01$) and the two first constrained axes explain 21.4% and 11.7% of the observed community variation, respectively. Treatment type is indicated by the symbol shape (round: control, triangle: glyphosate, square: Roundup), the hive origin by the symbol colour and the treatment dose by the symbol size. The black numbered circles indicate the most affected bacterial ASVs, and their corresponding identities are indicated in Table 1.

the control bees. This contrasting result between low and high Roundup exposure can be partly explained by genetic differences in bumblebees and differences in microbiome composition among hives in terms of their susceptibility to the Roundup. These results suggest that pure glyphosate modulates the microbiota by eradicating the dominant taxa skewing taxonomic abundance, but commercial glyphosate-based herbicides include co-formulants that are more noxious than glyphosate alone (Defarge et al. 2018, Helander et al. 2019, Mesnage et al. 2019, Straw et al. 2021).

Thus, the understanding of risks associated to glyphosate-based-herbicides requires considering the co-formulants of the commercial products. Glyphosate is always used within complex formulations. Each formulation includes both the active ingredient and co-formulants that facilitate the action of glyphosate. Co-formulants include surfactants that help the glyphosate penetrate leaves, emulsifiers helping products to stay mixed, and solvents to help dissolve the glyphosate in formulation (Straw et al. 2022). Unfortunately, the co-formulants in the formulants are often not listed in commercial products, which is also the case in our study. Previous studies have demonstrated high variation in the toxicity of pesticide formulations to bees (Mullin 2015, Mullin et al. 2015, Straw et al. 2021, 2022), and the effects of interactions between different co-formulants and active ingredients on bees is practically unstudied area.

The core gut microbiota of a healthy bumblebee is highly conserved and has a relatively low diversity, comprising only a few dominant taxa (Koch and Schmid-Hempel 2011, Hammer et al. 2021). In our study, the predominant taxa in the control bees were *Gilliamella*, *Snodgrasella alvi*, and *Candidatus Schmidhempelia*, commonly detected bacteria in bumblebee gut microbiota also in other bee studies (Kwong and Moran 2016, Hammer et al. 2021). The gut microbiota comprised mainly of dominant genera

Table 1. The most affected ASVs, as indicated by redundancy analysis (RDA) of the bacterial community explained by treatment, dose, and hive.

ID	Kingdom	Phylum	Class	Order	Family	Genus	Species
1	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Klebsiella*	NA
2	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Orbaceae	*Gilliamella*	NA
3	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Neisseriaceae	*Snodgrassella*	*alvi*
4	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Orbaceae	*Candidatus Schmidhempelia*	NA
5	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*apis*
6	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Orbaceae	*Candidatus Schmidhempelia*	NA
7	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Orbaceae	*Gilliamella*	*apicola*
8	Bacteria	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Saccharibacter*	*floricola*
9	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
10	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*bombicola*

The corresponding IDs and their positions in the RDA ordination are indicated in Fig. 2. The taxonomy was available for all 10 ASVs at the genus level and for 5 ASVs at the species level.

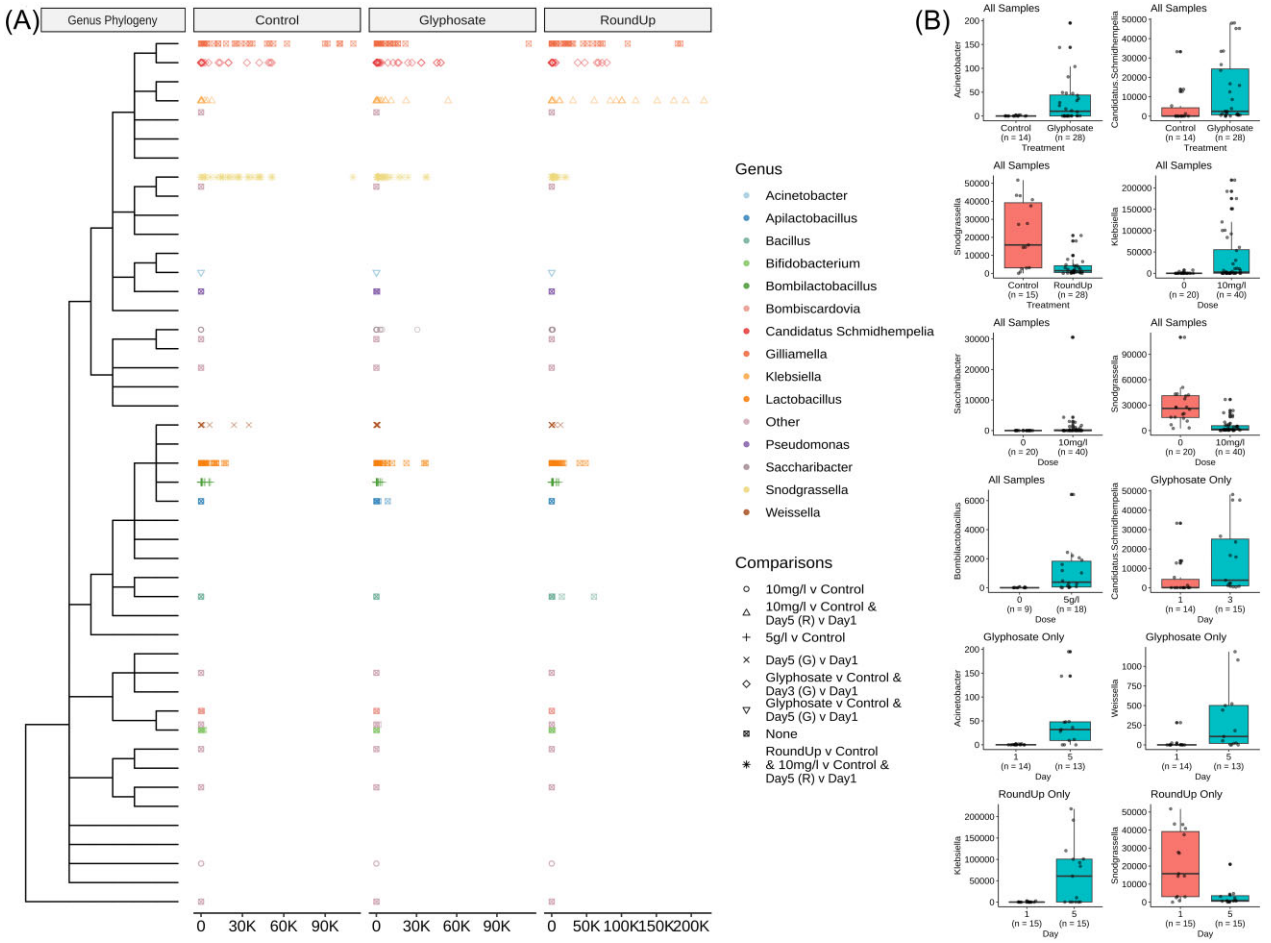

Figure 3. (A) ASV phylogeny at genus level indicating the number of observed reads for control, glyphosate, and Roundup treatments. The empty leaves indicate genera present in <10% of the samples. The symbol colour corresponds with taxonomic identity on the genus level, whereas the symbol shape indicates its significance in differential abundance comparisons ($P < .05$ after Benjamini–Hochberg-correction). (B) Twelve genera, including *Klebsiella*, *Gilliamella*, and *Snodgrasella*, exhibit significantly different abundances in response to glyphosate and Roundup exposure, the exposure dose, and exposure time ($P < .05$ after Benjamini–Hochberg-correction). Dose 0 = control; Day 1 = control. The comparisons include all glyphosate and Roundup samples irrespective of the dosage, dosage comparisons irrespective of the treatment type (with respective controls compared to the treatments) and temporal comparisons for the glyphosate and Roundup effects.

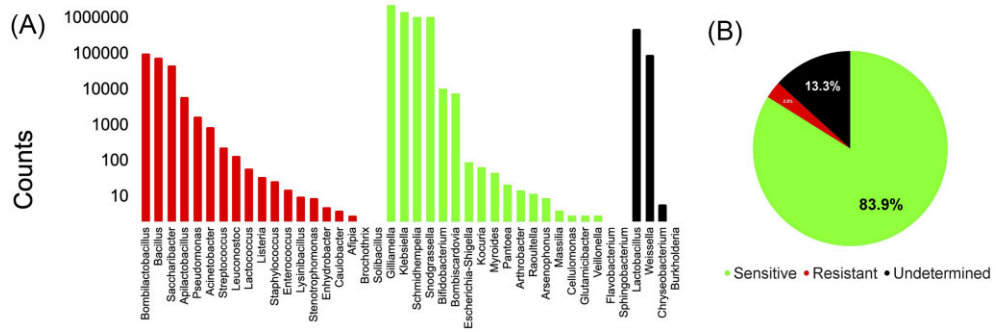

Figure 4. Distribution of bumblebee microbiota according to intrinsic sensitivity status to glyphosate. (A) Counts of bacterial ASVs that are potentially resistant (in red), sensitive (in green), and undetermined (in black) to glyphosate. (B) Percentage of resistant and sensitive bacteria to glyphosate.

Snodgrasella and *Gilliamella*, with a lower abundance of other taxa, such as *Weissella*, *Bifidobacterium*, and *Bombilactobacillus*. The dominant species varied among bee hives, probably due to the bacterial colonization history of hives. Bumblebee colonies are annual, and only the queen hibernates. Thus, each bumblebee hive has its own specific gut microbiota community (Fig. 2; Supplementary Fig. S3), which is initiated by the founder queen.

Our results suggest that, similar to other biological communities dominated by a few species, the decrease or extinction of the dominant species increases diversity. This was detected when exposing the bumblebees to glyphosate for 3 days. The gut microbiota diversity was further increased when the bees were fed 5 days of glyphosate-containing food. A novel bioinformatics tool based on the type of EPSPS enzyme produced in

microbes (Leino et al. 2021, Mathew et al. 2022) was used to test the potential susceptibility of bacterial ASVs to glyphosate. Thus, we propose that it is a useful tool for explaining some community shifts in microbiomes in response to glyphosate. Overall, members of the bee gut microbiota varied in susceptibility to glyphosate, largely corresponding to their EPSPS protein sequence class (Class I: sensitive to glyphosate; Class II: insensitive to glyphosate). Both glyphosate and glyphosate-based herbicide treatments significantly decreased the relative abundance of *S. alvi*, which, according to our bioinformatics approach, predominantly encodes the sensitive Class I. Accordingly, in experiments using eastern bumblebee (*B. impatiens*) or honey bees as target species, a decrease in *S. alvi* in gut microbiota has been detected after glyphosate treatment (Motta et al. 2018, Blot et al. 2019, Motta and Moran 2020, 2020, Castelli et al. 2021, 2022, 2023). Of the potentially glyphosate-resistant microbes having the EPSPS protein sequence Class II, the relative abundance of *Acinetobacter* genera increased when the bees were exposed to glyphosate in our experiments. *Weissella* and *Lactobacillus* genera, posing an unknown EPSPS glyphosate sensitivity class (Supplementary Table S1), increased in the bee gut after glyphosate treatment, thus leaving their glyphosate mode of action open for further studies. The classification of EPSPS enzymes failed to explain glyphosate sensitivity in only one case, even though other nontarget site mechanisms of resistance (Puigbò et al. 2022) are not considered in the analysis. Contrary to its glyphosate sensitive EPSPS protein sequence Class I (>1 million counts in the databases), the relative abundance of *Bombus* spp. specific *Candidatus Schmidhempelia* genera (Steele and Moran 2021) increased in bumblebees treated with glyphosate. This questions whether the bumblebees used in our study have altered EPSPS protein sequence compared to dominant *Candidatus Schmidhempelia* sequences, and what environmental pressures may have caused that.

The decrease in microbial diversity in bees exposed to a low but not higher exposure of Roundup remains to be solved in future studies. However, the lack of increased diversity suggests that commercial glyphosate-based herbicides include co-formulants that have negative effects on gut bacteria. Currently, only active ingredients (e.g. glyphosate) in herbicides are usually required to be tested for their toxicity to nontarget organisms (Mesnage et al. 2019). However, the test requirements of the co-formulants and products vary between regions (e.g. between EU and USA). Furthermore, the co-formulants in commercial products are regarded as confidential information and can vary geographically over time and between products. Healthy core microbiota have been shown to protect bumblebees from infection by the common parasite *Crithidia bombi* (Koch and Schmid-Hempel 2011). In honey bees, altered gut microbiota has been shown to reduce weight gain, change metabolism (Zheng et al. 2017), and increase mortality (Raymann et al. 2017, Dai et al. 2018, Castelli et al. 2021). In bumblebees, the gut microbiome has also been found to drive individual memory variation (Li et al. 2021). However, strong conclusions from our study should be warranted, because we used commercial laboratory raised bumblebee colonies, which may differ from wild bee colonies, e.g. in their gut microbiota composition and thus resistance and tolerance to microbial pathogens. Thus, we need more thorough studies on wild bumblebees and possible adverse effects of the heavy use of glyphosate-based herbicides on bumblebee health, pollination services, ecosystem functioning, and agricultural productivity.

Each foraging bumblebee makes trips several times a day to gather pollen and nectar. Bumblebees do not avoid glyphosate-treated plants and thus can be exposed to glyphosate and carry it back to the hive when they are foraging in recently sprayed fields (Thompson et al. 2022). Given that the plants and their flowers start to wither within 3–4 days of glyphosate-based herbicide spraying, the bee workers have ample opportunity for exposure to glyphosate-based herbicide in a day. Yet unanswered questions are (1) how the detected glyphosate and glyphosate-based herbicide-driven changes in bumblebee gut microbiota relate to the health, cognition, and behaviour of bees, and (2) whether they adversely affect pollination-dependent ecosystem functions and biodiversity loss.

Supplementary data

Supplementary data are available at FEMSEConline.

Author contributions

Marjo Helander (Conceptualization, Funding acquisition, Methodology, Project administration, Writing – original draft), Aditya Jeevannavar (Data curation, Formal analysis, Validation, Visualization, Writing – review & editing), Kimmo Kaakinen (Investigation, Methodology, Writing – review & editing), Suni A Mathew (Investigation, Methodology, Writing – review & editing), Kari Saikkonen (Conceptualization, Funding acquisition, Writing – review & editing), Benjamin Fuchs (Funding acquisition, Resources, Writing – review & editing), Pere Puigbò (Conceptualization, Formal analysis, Methodology, Software, Validation, Writing – review & editing), Olli J Loukola (Conceptualization, Resources, Writing – review & editing), and Manu Tamminen (Methodology, Supervision, Validation, Visualization, Writing – original draft)

Conflict of interest. None declared.

Funding

This work was supported by the Academy of Finland [grant number 311 077 to M.H. and 326 226 to K.S.]; Alhopuro Foundation to B.F.; and the Finnish Cultural Foundation to M.H.

Data availability

The DNA sequence data generated and analysed for this study can be found in the Sequence Read Archive under the BioProject ID PRJNA916876; http://www.ncbi.nlm.nih.gov/bioproject/916876.

References

Benbrook CM. Trends in glyphosate herbicide use in the United States and globally. *Environ Sci Eur* 2016;**28**:3. https://doi.org/10.1186/s12302-016-0070-0

Blot N, Veillat L, Rouzé R *et al.* Glyphosate, but not its metabolite AMPA, alters the honeybee gut microbiota. *PLoS One* 2019;**14**:e0215466. https://doi.org/10.1371/journal.pone.0215466

Callahan BJ, McMurdie PJ, Rosen MJ *et al.* DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;**13**:581–3. https://doi.org/10.1038/nmeth.3869

Caporaso JG, Lauber CL, Walters WA *et al.* Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA* 2011;**108**:4516–22. https://doi.org/10.1073/pnas.1000080107

Caporaso JG, Lauber CL, Walters WA *et al.* Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq

platforms. *ISME J* 2012;**6**:1621–4. https://doi.org/10.1038/ismej.2012.8

Carvell C, Bourke AFG, Dreier S *et al.* Bumblebee family lineage survival is enhanced in high-quality landscapes. *Nature* 2017;**543**:547–9. https://doi.org/10.1038/nature21709

Castelli L, Balbuena S, Branchiccela B *et al.* Impact of chronic exposure to sublethal doses of glyphosate on honey bee immunity, gut microbiota and infection by pathogens. *Microorganisms* 2021;**9**:845. https://doi.org/10.3390/microorganisms9040845

Chen IC, Hill JK, Ohlemüller R *et al.* Rapid range shifts of species associated with high levels of climate warming. *Science* 2011;**333**:1024–6. https://doi.org/10.1126/science.1206432

Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behavior. *Nat Rev* 2012;**13**:701–12. https://doi.org/10.1038/nrn3346

Cullen MG, Bliss L, Stanley DA *et al.* Investigating the effects of glyphosate on the bumblebee proteome and microbiota. *Sci Tot Environ* 2023;**864**:161074. https://doi.org/10.1016/j.scitotenv.2022.161074

Dai P, Yan Z, Ma S *et al.* The herbicide glyphosate negatively affects midgut bacterial communities and survival of honey bee during larvae reared *in vitro*. *J Agric Food Chem* 2018;**66**:7786–93. https://doi.org/10.1021/acs.jafc.8b02212

Defarge N, Spiroux de Vendômois J, Séralini GE. Toxicity of formulants and heavy metals in glyphosate-based herbicides and other pesticides. *Toxicol Rep* 2018;**5**:156–63. https://doi.org/10.1016/j.toxrep.2017.12.025

Dicks LV, Breeze TD, Ngo HT *et al.* A global-scale expert assessment of drivers and risks associated with pollinator decline. *Nat Ecol Evol* 2021;**5**:1453–61. https://doi.org/10.1038/s41559-021-01534-9

Ernst FGM, Shetty SA, Borman T *et al.* mia: Microbiome analysis. R package version 1.2.7. 2022. https://github.com/microbiome/mia (23 December 2022, date last accessed).

Fernandes AD, Macklaim JM, Linn TG *et al.* ANOVA-like differential expression (ALDEx) analysis for mixed population RNA-seq. *PLoS One* 2013;**8**:e67019. https://doi.org/10.1371/journal.pone.0067019

Ghazoul J. Buzziness as usual? Questioning the global pollination crisis. *Trends Ecol Evol* 2005;**20**:367–73. https://doi.org/10.1016/j.tree.2005.04.026

Goulson D, Nicholls E, Botías C *et al.* Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* 2015;**347**:1255957. https://doi.org/10.1126/science.1255957

Hallman CA, Sorg M, Jongejans E *et al.* More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS One* 2017;**12**:e0185809. https://doi.org/10.1371/journal.pone.0185809

Hammer TJ, Le E, Martin AN *et al.* The gut microbiota of bumblebees. *Insectes Soc* 2021;**68**:287–301. https://doi.org/10.1007/s00040-021-00837-1

Helander M, Lehtonen TK, Saikkonen K *et al.* Field-realistic acute exposure to glyphosate-based herbicide impairs fine-color discrimination in bumblebee. *Sci Tot Environ* 2023;**857**:159298. https://doi.org/10.1016/j.scitotenv.2022.159298

Helander M, Pauna A, Saikkonen K *et al.* Glyphosate residues in soil affect crop plant germination and growth. *Sci Rep* 2019;**9**:19653. https://doi.org/10.1038/s41598-019-56195-3

Koch H, Schmid-Hempel P. Socially transmitted gut microbiota protect bumblebees against an intestinal parasite. *Proc Natl Acad Sci USA* 2011;**108**:19288–92. https://doi.org/10.1073/pnas.1110474108

Kwong WK, Moran NA. Gut microbial communities of social bees. *Nat Rev Microbiol* 2016;**14**:374–84. https://doi.org/10.1038/nrmicro.2016.43

Lee W-J, Hase K. Gut microbiota–generated metabolites in animal health and disease. *Nat Chem Biol* 2014;**10**:416–24. https://doi.org/10.1038/nchembio.1535

Leino L, Tall T, Helander M *et al.* Classification of the glyphosate target enzyme (5-enolpyruvylshikimate-3-phosphate synthase) for assessing sensitivity of organisms to the herbicide. *J Hazard Mater* 2021;**408**:124556. https://doi.org/10.1016/j.jhazmat.2020.124556

Li L, Solvi C, Zhang F *et al.* Gut microbiome drives individual memory variation in bumblebees. *Nat Commun* 2021;**12**:6588. https://doi.org/10.1038/s41467-021-26833-4

Lin H, Eggesbo M, Peddada SD. Linear and nonlinear correlation estimators unveil undescribed taxa interactions in microbiome data. *Nat Commun* 2022;**13**:1–16. https://doi.org/10.1038/s41467-022-32243-x

Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;**15**:550. https://doi.org/10.1186/s13059-014-0550-8

Lowder SK, Skoet J, Raney T. The number, size, and distribution of farms, smallholder farms, and family farms worldwide. *World Dev* 2016;**87**:16–29. https://doi.org/10.1016/j.worlddev.2015.10.041

Mallick H, Rahnavard A, McIver LJ *et al.* Multivariable association discovery in population-scale meta-omics studies. *PLoS Comput Biol* 2021;**17**:e1009442. https://doi.org/10.1371/journal.pcbi.1009442

Mathew SA, Muola A, Saikkonen K *et al.* Quantification of the potential impact of glyphosate-based products on microbiomes. *J Vis Exp* 2022;**179**:e63109. https://doi.org/10.3791/63109

Mesnage R, Benbrook C, Antoniou MN. Insight into the confusion over surfactant co-formulants in glyphosate-based herbicides. *Food Chem Toxicol* 2019;**28**:137–45. https://doi.org/10.1016/j.fct.2019.03.053

Motta EVS, Mak M, De Jong TK *et al.* Oral or topical exposure to glyphosate in herbicide formulation impacts the gut microbiota and survival rates of honey bees. *Appl Environ Microbiol* 2020;**86**:e01150–20. https://doi.org/10.1128/AEM.01150-20

Motta EVS, Moran NA. Impact of glyphosate on the honey bee gut microbiota: effects of intensity, duration, and timing of exposure. *mSystems* 2020;**5**:e00268–20. https://doi.org/10.1128/mSystems.00268-20

Motta EVS, Moran NA. The effects of glyphosate, pure or in herbicide formulation, on bumble bees and their gut microbial communities. *Sci Tot Environ* 2023;**872**:162102. https://doi.org/10.1016/j.scitotenv.2023.162102

Motta EVS, Powell JE, Moran NA. Glyphosate induces immune dysregulation in honey bees. *Anim Microbiome* 2022;**4**:16. https://doi.org/10.1186/s42523-022-00165-0

Motta EVS, Raymann K, Moran NA. Glyphosate perturbs the gut microbiota of honey bees. *Proc Natl Acad Sci USA* 2018;**115**:10305–10. https://doi.org/10.1073/pnas.1803880115

Mullin CA. Effects of 'inactive' ingredients on bees. *Curr Opin Insect Sci* 2015;**10**:194–200. https://doi.org/10.1016/j.cois.2015.05.006

Mullin CA, Chen J, Fine JD *et al.* The formulation makes the honey bee poison. *Pestic Biochem* 2015;**120**:27–35. https://doi.org/10.1016/j.pestbp.2014.12.026

Oksanen J, Simpson GL, Blanchet FG *et al.* Vegan: Community Ecology Package. R package version 2.6-2. 2022. https://CRAN.R-project.org/package=vegan (23 December 2022, date last accessed).

Potts SG, Biesmeijer JC, Kremen C *et al.* Global pollinator declines: trends, impacts and drivers. *Trends Ecol Evol* 2010;**25**:345–53. https://doi.org/10.1016/j.tree.2010.01.007

Potts SG, Imperatriz-Fonseca VL, Ngo HT *et al.* Safeguarding pollinators and their values to human well-being. *Nature* 2016;**540**:220–9. https://doi.org/10.1038/nature20588

Puigbò P, Leino L, Rainio MJ *et al.* Does glyphosate affect the human microbiota? *Life* 2022;**12**:707. https://doi.org/10.3390/life12050707

Rainio M, Ruuskanen S, Helander M *et al.* Adaptation of bacteria to glyphosate: a microevolutionary perspective of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase. *Environ Microbiol Rep* 2021;**13**:309–16. https://doi.org/10.1101/2020.06.16.154005

Raymann K, Shaffer Z, Moran NA. Antibiotic exposure perturbs the gut microbiota and elevates mortality in honey bees. *PLoS Biol* 2017;**15**:e2001861. https://doi.org/10.1371/journal.pbio.2001861

Reilly JR, Artz DR, Biddinger D *et al.* Crop production in the USA is frequently limited by a lack of pollinators. *Proc R Soc B* 2020;**287**:20200922. https://doi.org/10.1098/rspb.2020.0922

Rooks MG, Garrett WS. Gut microbiota, metabolites and host immunity. *Nat Rev Immunol* 2016;**16**:341–52. https://doi.org/10.1038/nri.2016.42

Ruuskanen S, Fuchs B, Nissinen R *et al.* Ecosystem consequences of herbicides: the role of microbiome. *Trends Ecol Evol* 2023;**38**:35–43. https://doi.org/10.1016/j.tree.2022.09.009

Ruuskanen S, Rainio M, Gomez-Gallego C *et al.* Glyphosate-based herbicides influence antioxidants, reproductive hormones and gut microbiome but not reproduction: a long-term experiment in an avian model. *Environ Pollut* 2020;**266**:115108. https://doi.org/10.1016/j.envpol.2020.115108

Schmid-Hempel R, Schmid-Hempel P. Female mating frequencies in *Bombus* spp. from Central Europe. *Insectes Soc* 2000;**47**:36–41. https://doi.org/10.1007/s000400050006

Steele MI, Moran NA. Evolution of interbacterial antagonism in bee gut microbiota reflects host and symbiont diversification. *mSystems* 2021;**6**:e00063–21. https://doi.org/10.1128/mSystems.00063-21

Straub D, Blackwell N, Langarica-Fuentes A *et al.* Interpretations of environmental microbial community studies are biased by the selected 16S rRNA (gene) amplicon sequencing pipeline. *Front Microbiol* 2020;**11**:550420. https://doi.org/10.3389/fmicb.2020.550420

Straw EA, Carpentier EN, Brown MJF. Roundup causes high levels of mortality following contact exposure in bumble bees. *J Appl Ecol* 2021;**58**:1167–76. https://doi.org/10.1111/1365-2664.13867

Straw EA, Thompson LJ, Leadbeater E *et al.* 'Inert' ingredients are understudied, potentially dangerous to bees and deserve more research attention. *Proc R Soc B* 2022;**289**:20212353. https://doi.org/10.1098/rspb.2021.2353

Tang Q-H, Li W-L, Wang J-P *et al.* Effects of spinetoram and glyphosate on physiological biomarkers and gut microbes in *Bombus terrestris*. *Front Physiol* 2023;**13**:1054742. https://doi.org/10.3389/fphys.2022.1054742

Teseo S, van Zweden JS, Pontieri L *et al.* The scent of symbiosis: gut bacteria may affect social interactions in leaf-cutting ants. *Anim Behav* 2019;**150**:239–54. https://doi.org/10.1016/j.anbehav.2018.12.017

Thompson LJ, Smith S, Stout JC *et al.* Bumblebees can be exposed to the herbicide glyphosate when foraging. *Environ Toxicol Chem* 2022;**41**:2603–12. https://doi.org/10.1002/etc.5442

Zattara EE, Aizen MA. Worldwide occurrence records reflect a global decline in bee species richness. *One Earth* 2021;**4**:114–23. https://doi.org/10.1016/j.oneear.2020.12.005

Zheng H, Powell JE, Steele MI *et al.* Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. *Proc Natl Acad Sci USA* 2017;**114**:4775–80. https://doi.org/10.1073/pnas.1701819114

Zhou H, He K, Chen J *et al.* LinDA: linear models for differential abundance analysis of microbiome compositional data. *Genome Biol* 2021;**23**:95. https://doi.org/10.1186/s13059-022-02655-5

Received 6 April 2023; **revised** 26 May 2023; **accepted** 13 June 2023