Glyphosate contamination: implications for honeybee *Apis mellifera* and consumers in Southeastern Mexico

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ABSTRACT

Objective: in a study conducted from June 2021 to May 2022 in two apiaries in southeastern Mexico, levels of glyphosate residues in pollen collected by bee *Apis mellifera* were analyzed to assess potential risks to both bees and humans.

Design/methodology/approach: the analysis used an immunoassay method after residue extraction using the QUECHERS method.

Results: the results revealed the presence of glyphosate in all samples, with concentrations ranging between 3.71 and 7.29 μg kg^{-1}. However, risk analysis, as indicated by the pollen hazard quotient, suggested that these quantities did not pose a serious threat to bees or humans. The levels were within the limits of the acceptable daily intake (ADI), the acute reference dose (ARfD) and the acceptable operator exposure level (AOEL).

Limitations/implications: although this study did not find any significant association between glyphosate and potential risks for both humans and bees, its persistence in the environment was demonstrated.

Findings/conclusions: Glyphosate levels at the study site were low, suggesting minimal risk to both humans and bees. However, the wide distribution of glyphosate in the region makes it necessary to emphasize long-term studies to understand the possible chronic effects of the pesticide on all species in the area.

Keywords: *Apis mellifera*, transgenic crops, herbicide, pollen hazard quotient.

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INTRODUCTION

For ease in weed control management, specific crops have been genetically altered to resist the herbicide glyphosate (GLY), resulting in a heightened global use of this wide-ranging systemic herbicide (Green, 2018; Székács & Darvas, 2012). GLY operates by blocking the activity of 5-enolpyruvylshikimate-3-phosphate synthase, an enzyme crucial for the biosynthesis of aromatic amino acids in plants (Duke & Powles, 2008; Shilo *et al.*,

2016). Because animals lack this enzyme, GLY is generally considered one of the least toxic pesticides for them (Duke & Powles, 2008). However, there is evidence indicating that GLY affects organisms beyond plants. For example, it has been demonstrated to decrease the reproduction of earthworms residing in the soil (Gaupp-Berghausen *et al.*, 2015). Moreover, GLY influences the growth of microalgae and aquatic bacteria and has detrimental effects on fish, amphibians, mammals, and birds (Benachour & Séralini, 2009; Relyea, 2005; Richard *et al.*, 2005). GLY is additionally associated with adverse effects on soil rhizosphere-associated bacterial communities (Newman *et al.*, 2016) and a decrease in mycorrhizal colonization (Helander *et al.*, 2018). Bees, as insects with a heightened risk of exposure, are especially susceptible due to various pathways of contact. They may encounter GLY while gathering nectar and pollen from flowering plants located several kilometers away, and subsequently transport any contaminants from these sources back to the hive (Agrebi *et al.*, 2019; Coupe *et al.*, 2012; Krupke *et al.*, 2012). The main routes of exposure involve interactions with agricultural crops, drift, and the widespread application of glyphosate formulations in urban environments for household and minor non-agricultural activities, such as weed control along railways, in parks, and within home gardens (Pasquale *et al.*, 2013; Silva *et al.*, 2018; Simon-delso *et al.*, 2017). Pesticide residues have been observed in a variety of bee-derived products, such as honey, pollen, propolis, wax, royal jelly, and honeycomb (Calatayud-Vernich *et al.*, 2017; de Oliveira *et al.*, 2016; Matin *et al.*, 2016; Pohorecka *et al.*, 2012; Ruiz-Toledo *et al.*, 2018; Tosi *et al.*, 2018; Valdovinos-Flores *et al.*, 2017; Zawislak *et al.*, 2019). Nevertheless, since 1990, the introduction and rapid spread of herbicide-resistant crops globally, including in Mexico (James, 2016), have led to increased GLY application and, consequently, to higher health risks for both honeybees and consumers (Agrebi *et al.*, 2019; Bohan *et al.*, 2005; Foulk, 2009; Rubio *et al.*, 2014).

Although GLY may not show significant toxicity to adult bees (Lewis *et al.*, 2016), concerns have been raised regarding potential chronic effects due to the accumulation of pesticide residues within beehives (Boily *et al.*, 2013; Crenna *et al.*, 2020; Herbert *et al.*, 2014; Weisbrod, 2020; J. Wu *et al.*, 2012; Zawislak *et al.*, 2019). For example, young adult bees exposed chronically to glyphosate formulations have displayed impaired associative learning and reduced sensitivity to sucrose (Gonalons & Farina, 2018; Herbert *et al.*, 2014; Luo *et al.*, 2021). Additionally, forager bees exposed to sublethal doses of such formulations have shown difficulties in navigating back to their hives (Balbuena *et al.*, 2015).

Concerning human health, numerous regulatory agencies and scientific organizations worldwide have reached a consensus that there is no conclusive evidence suggesting that glyphosate causes health problems (EFSA, 2015b; European Commission, 2002; USEPA, 1993). However, there is evidence indicating that residues of glyphosate found in the environment could potentially pose health risks to humans (Agrebi *et al.*, 2019). These risks include teratogenic, tumorigenic, and hepatorenal effects, which have been associated with endocrine disruption and oxidative stress, leading to metabolic alterations. The risk of exposure is further heightened by the fact that bee products are a part of the human supplementary diet. For instance, pollen is often considered an excellent dietary supplement for nutrition and is available in various forms on the market, such as granules,

capsules, tablets, and powders (Komosinska-Vassev *et al*., 2015; Kostić *et al*., 2020). This potentially amplifies the risk to human health.

In our study area, beekeepers typically place their beehives in uncultivated areas that allow for natural plant succession. However, these locations are often surrounded by a landscape featuring various crops such as soybean, mango, beans, pumpkins, maize, and sesame (as observed by the authors). While soybean is the only genetically modified crop in this region, non-transgenic varieties are also cultivated. Consequently, foraging honeybees potentially encounter a range of pesticides, including GLY, in water, pollen, and nectar (Hladik *et al*., 2016; Krupke *et al*., 2012). Moreover, since many farmers have transitioned from non-transgenic to transgenic soybean varieties, it is assumed that the use of this herbicide has increased. As a result, transgenic soybean likely plays a significant role in the contamination by GLY. Therefore, we hypothesized that colonies located in areas where transgenic soybean pollen is present would exhibit higher levels of GLY. The objective of our study was to quantify glyphosate residues in pollen samples collected from honeybees (*Apis mellifera* L.) and assess the potential risk it may pose to honeybees and the health of consumers.

MATERIALS AND METHODS

Study period, site and sample collection

The study was carried out in the municipalities of Suchiate and Tapachula, in the Soconusco region, Chiapas, in southern Mexico. We selected two sites based on different land uses: Site 1 (14° 45′5.08″N, 92° 15′46.87″W) in Suchiate, characterized by 2% urban settlements, 36% preserved remnants of the original forest, and 62% cropland; and Site 2 (14° 45′19.20″N, 92° 17′30.60″W) in Tapachula, with 1% of land occupied by urban settlements, 17% covered by the original forest, and 82% designated as cropland. To minimize the influence of geographical factors, the sampling sites were located within the same ecoregion and separated by 3 km (Figure 1). We operated under the assumption that the foraging areas of the bees were relatively independent and restricted to their respective sites, given the perceived adequacy of food resources near the hives, as indicated by honey production levels. Throughout one year, we gathered monthly pollen samples from ten colonies of *A. mellifera* at each site, totaling 120 pollen samples. Each sample was preserved in a 15 mL Falcon tube and kept frozen at −20 °C until analysis. Simultaneous sampling was conducted at both sites.

Glyphosate extraction and quantification

GLY residues were extracted using the methodology developed by Wiest *et al*. (2011). Two grams of pollen were accurately weighed and placed in a 50 mL centrifuge tube. To this, 10 mL of water were added, and the mixture was vigorously shaken. Subsequently, 10 mL of acetonitrile, 3 mL of hexane, 4 g of anhydrous $MgSO_4$, 1.0 g of sodium chloride, 1 g of sodium citrate dihydrate, and 500 mg of disodium citrate sesquihydrate were added. The tube was promptly shaken by hand, vortexed for one minute, and then centrifuged for 2 minutes at 5000 g. Six milliliters of the supernatant were carefully transferred into a 15 mL PSA (primary secondary amine) tube, which contained 900 mg

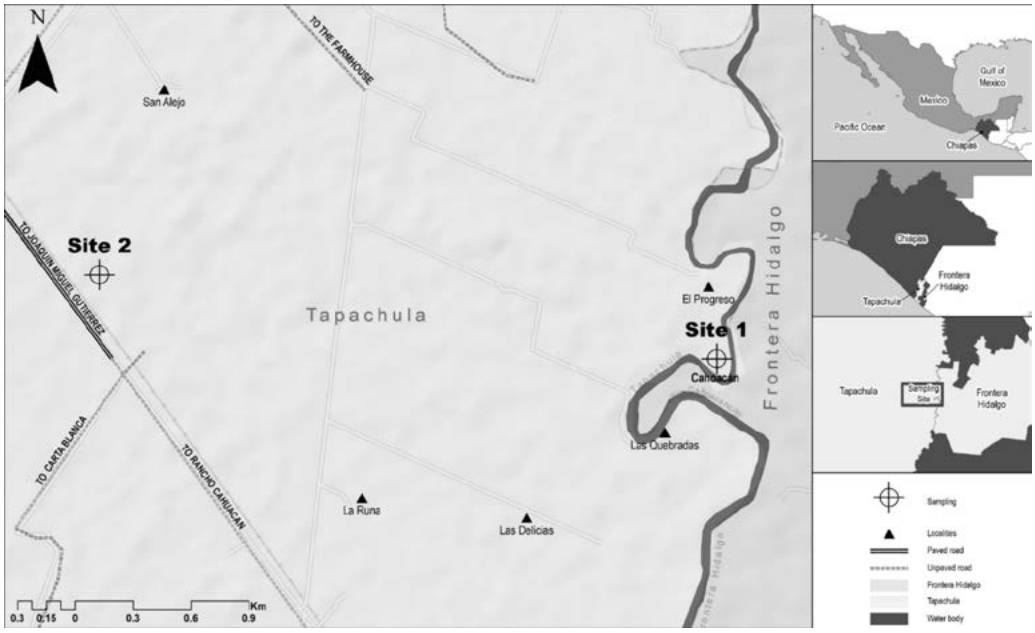

Figure 1. Sampling sites in the study. Sites were separated by approximately 3 km to keep foragers from visiting same resources.

of anhydrous $MgSO_4$, 150 mg of PSA bonded silica, and 150 mg of C18 bonded silica. This tube was immediately shaken by hand, vortexed for 10 seconds, and centrifuged for 2 minutes at 5000 g. Finally, 4 mL of the extract were transferred into a 10 mL glass, cone-ended centrifuge tube, evaporated until a final volume of 50 μL was achieved, and the sample was stored at −18 °C until analysis. All salts used for extraction were supplied by Agilent Technologies, Santa Clara, CA, USA.

GLY quantification was performed using an immunoassay kit from Abraxis LLC (Part number PN500084: Warminster, USA). The method demonstrated a limit of detection for GLY of 0.05 μg/L, a limit of quantification of 0.13 μg/L, a maximum detectable concentration of 4 μg/L, and an average recovery rate of 102%. For quantification, a four-point calibration curve was established (0.075, 0.2, 0.75, and 4 μg/L) with two replicates for each point. An analytical quality control solution at 0.5 μg/L was also employed. All samples underwent triplicate analysis, and for samples with GLY concentrations exceeding the calibration curve range, dilutions were performed until a reliable concentration estimate could be obtained. Possible cross-reactivity with other agrochemicals used in the study area, such as paraquat, spinosad, malathion, mancozeb, endosulfan, chlorothalonil, chlorpyrifos, and cypermethrin, was investigated. No interference was observed in the analysis, as confirmed by control tests in which these pesticides, at a concentration of 1 μg/L, did not react with our immunoassay test (Ruiz-Toledo *et al.*, 2014).

Exposure assessment and risk characterization to honeybee health

We calculated the average concentration of GLY found in the replicates and determined the Pollen Hazard Quotient (PHQ), which is a measure of the number of bees that, by consuming one kilogram of pollen, would reach the LD50 (dose required to kill 50% of

the exposed population). To calculate the PHQ for honeybees, we followed the methods described by Stoner and Eitzer (2013a) and Traynor *et al.* (2016). This was done by dividing the concentration (μg/kg) of GLY in the sample by the oral LD50 of GLY (100 μg/bee), which is generally regarded as moderately toxic for adult bees (EFSA, 2015a; Lewis *et al.*, 2016) and then multiplying by 100. For reference, an adult bee that consumed 100 mg of pollen with a PHQ of 1000 would have ingested approximately 10% of the LD50 of the pesticide during its development stage as a nurse bee, which lasts about 10 days (Calatayud-Vernich *et al.*, 2018). If this 10% of the LD50 should not be exceeded (Atkins *et al.*, 1981), a PHQ value of 1000 would correspond to a critical threshold for bee health (Stoner & Eitzer, 2013b; Traynor *et al.*, 2016). A nurse bee typically consumes between 13 and 120 mg of pollen during its first 10 days of life (OECD/OCDE, 1998; Rortais *et al.*, 2005), with an average consumption of 65 mg (M.-P. Chauzat & Faucon, 2007). As a worst-case scenario, we considered the maximum consumption level of 12 mg of pollen per day (Rortais *et al.*, 2005). We then multiplied this highest consumption level by the highest observed glyphosate residues and compared the resulting exposure levels with the oral acute LD50 of GLY.

Risk to consumer's health

The toxicological reference values for GLY in this study were: 1) the acceptable daily intake (ADI) at 0.3 mg/kg bodyweight (Renwick, 2002a); 2) the daily acute reference dose (ARfD) at 0.5 mg/kg bodyweight (EFSA, 2015b); 3) the legally permitted maximum concentration of pesticide residues in or on food products or animal feed (MRL) at 0.05 mg/kg (EFSA, 2017); and 4) the Acceptable Operator Exposure Level (AOEL) at 0.1mg/kg (EFSA, 2015a; Luo *et al.*, 2021; Renwick, 2002b).

To assess the health risk associated with GLY residues in pollen for consumers, we relied on pollen consumption estimates obtained from data published in the EFSA Comprehensive European Food Consumption Database (EFSA, 2018). The highest 95th percentile value recorded corresponds to 69.55 g/person, which equates to 1.35 g/kg bodyweight for a person weighing 52 kilograms in France. In the most conservative scenario, we multiplied such high intake levels by the highest observed concentration of GLY residues. Finally, we compared the resulting exposure levels with the established toxicological reference values for GLY to determine the extent of the risk.

Statistical analysis

A descriptive analysis of GLY concentration was performed, which included calculating the geometric means, median, standard deviations, as well as minimum and maximum values. To identify any statistical differences among different sampling dates and between the two sites, a general linear mixed model ANOVA was conducted. In this analysis, the colony was treated as a random effect, while the site and the date of sampling were considered fixed effects. All statistical analyses were carried out using the R software package, and the significance level was set at 0.05 (R Development Core Team, 2020).

RESULTS AND DISCUSSION

Glyphosate residues in pollen

We detected GLY residues in all samples in the range of 3.71 to 7.29 μg/kg (Table 1). In site 1, a mean (±SD) GLY concentration of 5.07 μg/kg (±0.93) was found, while in site 2 it was 5.45 μg/kg (±0.84). No statistically significant differences were observed between sites ($p \geq 0.05$). The highest GLY concentration was found in site 1 in March (7.29 μg/kg). However, no significant difference was found between months ($p \geq 0.05$). In our study area, GLY residues were identified in all samples collected at both sites (site 1, 3.9 - 7.29 μg/kg and site 2, 3.71 - 6.68 μg/kg). Residues of pesticides, including GLY, have been identified in live honeybees, stored fresh pollen, and beeswax. Notably, the beeswax contains elevated levels of commonly employed acaricides in beekeeping (Kasiotis *et al.*, 2023). Thompson *et al.* (2014) identified this herbicide in brood samples at concentrations ranging from 1.23 to 19.5 mg/kg; Rubio *et al.* (2014) reported a mean concentration of 64 mg/kg and a maximum of 163 mg/kg in honey; Agrebi *et al.* (2019) found glyphosate residues in 91.4% of the bee bread samples, whose main component is pollen; the average concentration reported in this study is 55.52 mg/kg, seven times higher than in our study.

Exposure assessment and risk characterization of GLY residues in pollen for honeybees

In the estimation of HQ for GLY residues in pollen, we found an average of 5.79 for site 1 and 5.45 for site 2. This indicates that at site 1 an adult bee consumes 0.06% of the LD50 during its development stage, while in site 2 it is 0.05% (Table 1). In the worst case and with the maximum concentration of residues detected in pollen, we found that these concentrations could correspond to doses of 0.87 μg of GLY residues ingested per nurse

Table 1. Concentration of GLY in pollen (μg/Kg) in the samples from both study sites (S1 and S2); N, number of samples; [a] % of samples with detectable levels (%≥DL); [b] values reported as geometric mean (GM); (SD) standard deviation; (HQ) Hazard quotient; % of LD50 refers to the proportion of the LD50 ingested daily by a bee.

Year	Month	N	%≥DL [a]		GM [b]		Median		SD		HQ		% of LD50	
			S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
2021	June	10	100	100	5.03	6.02	6.14	5.88	1.15	0.36	5.03	6.02	0.05	0.06
	July	10	100	100	6.54	6.68	6.54	6.09	0.45	1.14	6.54	6.68	0.07	0.07
	August	10	100	100	7.29	6.09	6.47	5.06	0.68	1.21	7.29	6.09	0.07	0.06
	September	10	100	100	6.47	4.48	6.51	4.48	0.34	0.68	6.47	4.48	0.06	0.04
	October	10	100	100	5.93	3.71	6.14	5.29	0.32	1.14	5.93	3.71	0.06	0.04
	November	10	100	100	6.57	5.06	5.81	5.06	1.45	0.60	6.57	5.06	0.07	0.05
	December	10	100	100	6.34	5.98	6.46	5.98	1.20	0.74	6.34	5.98	0.06	0.06
2022	January	10	100	100	3.95	4.86	4.94	4.86	0.98	0.71	3.95	4.86	0.04	0.05
	February	10	100	100	4.94	6.26	5.91	6.26	0.57	0.60	4.94	6.26	0.05	0.06
	March	10	100	100	5.91	5.78	6.57	6.02	0.69	0.16	5.91	5.78	0.06	0.06
	April	10	100	100	6.35	5.66	6.35	5.72	0.70	0.49	6.35	5.66	0.06	0.06
	May	10	100	100	5.07	5.69	5.91	5.94	0.72	0.16	5.07	5.69	0.05	0.06

bee over 10 days (0.012 g × 7.29 μg/kg × 10 days). This exposure level corresponds to approximately 0.008% of the oral LD50 of GLY.

Based on our assessments, the GLY concentrations found here do not appear to be toxic to bees. The maximum concentration of GLY residues found (7.29 μg/kg) led to sub-lethal exposure (not acutely toxic to bees), equivalent to a dose of 0.87 μg/bee (0.008% of its LD50), ingested over the first 10 days of life of a nurse bee. Nevertheless, some studies have suggested that even minute amounts of GLY may compromise the immune system of bees (Samsel & Seneff, 2013). Studies have shown that it increases the susceptibility of bees to the effects of other pesticides (J. Y. Wu *et al.*, 2011), perhaps working as a synergist (Botías *et al.*, 2017; A. M. Chauzat *et al.*, 2009; Wan *et al.*, 2018). This phenomenon has been observed with some fungicides which increases the toxicity of pyrethroids (Pilling *et al.*, 1995; Pilling & Jepson, 1993), and of some neonicotinoids (Iwasa *et al.*, 2004) to the honeybee. Furthermore, GLY, as well as the herbicide formulation containing GLY, can affect the intestinal microbiota of bees, leading to dysbiosis and increased susceptibility to bacterial infection (Motta *et al.*, 2020, 2022). GLY exposure has been found to decrease the expression of genes encoding antimicrobial peptides and inhibit melanization, which are important components of the bee's innate immune system (Vázquez *et al.*, 2018). Additionally, GLY exposure may disrupt the beneficial intestinal microbiota of bees, potentially affecting bee health and their effectiveness as pollinators (Motta *et al.*, 2018). The effects of GLY on bee health and intestinal microbiota may vary depending on individual and colony susceptibility (Helander *et al.*, 2018, 2023)

Risk assessment for consumers to contaminated pollen

According to our results, a high pollen consumption in southeastern Mexico could lead to a daily ingestion of 0.005 μg GLY/kg bodyweight. No sample in our study exceeded the toxicological reference values. Rubio *et al.* (2014) found GLY residues in 70% of honey samples from countries that permitted the cultivation of genetically modified organisms, compared to only 21% in those that did not. In our study, we identified GLY residues in 100% of the pollen samples, indicating that contamination by this herbicide extends beyond honey, aligning with findings reported in soybeans, cereals, and ice cream (IARC, 2017; Kolakowski *et al.*, 2020; Rubio *et al.*, 2014; Vicini *et al.*, 2021). While tolerance levels for GLY and its metabolites have been established in various foods (Code of Federal Regulations., 2018), none have been set for pollen and honey. Mexico lacks federal monitoring programs for GLY, making it challenging to estimate the extent of food contamination in the country. In contrast, countries like the USA include GLY in their annual pesticide residue-monitoring program, detecting it in various commodities (FDA, 2017). The Canadian Food Inspection Agency reported GLY in food, with a presence in 29.7% of 3,188 analyzed food samples in 2015-2016.

GLY is recognized as a public health risk, prompting global concern and social action, especially since it has entered the human food chain (Mills *et al.*, 2017). Recent research indicates an increase in the prevalence and average concentration of GLY in human

urine between 1993 and 2016 (Philipp Schledorn, 2014). Possible mechanisms underlying GLY toxicity in mammals have been described in recent studies (Mensah *et al.*, 2015). The 2016 report from the International Agency for Research on Cancer of the World Health Organization (IARC, 2017) summarizes scientific data, and based on that report, the state of California in the USA listed GLY as known to cause cancer, requiring products to be labeled accordingly (California Environmental Protection Agency, 2017). Moreover, other studies have reported that the toxicological effects of GLY depend on the type of cells, chemical composition, as well as the magnitude and time of exposure (Agostini *et al.*, 2020). This includes neurological effects (Martinez & Al-Ahmad, 2019), damage to the immune system (Santovito *et al.*, 2018), effects on human embryonic and placental cells (Benachour & Séralini, 2009; Richard *et al.*, 2005) and decreased sperm motility, viability, and mitochondrial activity (Nerozzi *et al.*, 2020). It has also been noted that even at concentrations below toxic levels (<1 μg/L), GLY can reduce testosterone production by 35% and disrupt estrogen-regulated genes, promoting breast tumor growth (Hokanson *et al.*, 2007). This situation is concerning, particularly since the Soconusco region has shown GLY residues in various matrices for human consumption (honey and well water). Although the concentrations of GLY reported in our studies did not appear to pose a high risk, a recent increase in the incidence of kidney problems, reproductive issues, cancer, and leukemia in the region has been reported, potentially associated with the unregulated use of this herbicide (Rivera-luna *et al.*, 2014).

CONCLUSIONS

The detection of GLY in pollen collected by honeybees raises concerns about potential contamination in food supplies for both humans and bees. Nevertheless, despite the presence of GLY residues in all samples analyzed in this study, the reported concentrations do not seem to pose any apparent risk to the health of humans and bees, as per our calculations and considering the LD50. However, given the widespread distribution of GLY in the region, it is imperative to underscore the importance of conducting long-term studies to comprehend the potential chronic effects of the pesticide on all species in the area.

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