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Acute 24h oral LC_{50} of $CuSO_4$ in newly emerged worker honey bees (*Apis mellifera*)

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Abstract

Honey bees (*Apis mellifera*) are essential pollinators for ecosystem functioning and agricultural production; however, agrochemicals and environmental contaminants can pose acute and chronic risks to bee health. Here, we assessed the 24 h acute oral toxicity of copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), widely used in agriculture, in newly emerged (0-day-old) worker honey bees under laboratory conditions and estimated lethal concentration (LC) values using probit regression. Newly emerged workers from a single colony were used; bees were housed in plastic cages (10 bees per cage) and maintained at 25 ± 2 °C and ≥50% relative humidity. Throughout the experiment, bees were fed ad libitum with 50% (w/v) sucrose solution. Fresh $CuSO_4 \cdot 5H_2O$ solutions were prepared daily in 50% (w/v) sucrose solution. A range finding test screened 50–10,000 ppm, and the definitive test included 4500, 5000, 5250, 5500, 5750, 6000, 6500, and 7000 ppm to bracket the LC_{50} region. Each concentration and the control were tested in triplicate (27 cages in total), and mortality was recorded hourly. Mortality data were analysed using probit regression in IBM SPSS Statistics 29.0. The estimated 24 h LC_{50} was 4711 ppm (95% CI: 4309–4952). The LC_5, LC_{10}, LC_{20}, LC_{50} and LC_{90} values were 3006, 3383, 3839, 4711 and 6040 ppm, respectively. Mortality increased consistently with concentration, indicating a clear acute dose–response relationship for $CuSO_4 \cdot 5H_2O$. The findings presented herein contribute quantitative baseline data for the risk assessment of copper-based agrochemicals with respect to acute oral exposure in newly emerged worker honey bees.

Keywords: Acute oral toxicity, *Apis mellifera*, Copper sulfate, LC_{50}, Probit analysis

INTRODUCTION

Honey bees (*Apis mellifera*) are keystone pollinators of wild ecosystems and agricultural crops (Rasuli et al. 2015, Potts et al. 2010). Honey bees are critical for pollinating many economically important crops, as efficient pollen transfer is essential for optimal seed set and fruit development. Over the last few years, however, there have been alarming declines in honey bee colonies (Goulson et al. 2015, Potts et al. 2010). These declines are believed to be due to the combined effects of various stressors such as exposure to pesticides, habitat loss, pathogens and environmental contamination (Goulson et al. 2015). Honey bees may be exposed to a wide range of environmental contaminants as they forage over large areas. In a survey of North American beehives, residues of 121 agrochemicals were found in hive-associated matrices (bees, pollen and beeswax) (Mullin et al. 2010).

Metal contamination from agricultural and industrial practices, along with agrochemicals, poses a less visible threat. Although metals are naturally present in the environment, anthropogenic inputs may elevate metal concentrations in soils and vegetation, which bees can collect through nectar and pollen (Di et al. 2016). Also, elevated concentrations of metals such as lead (Pb), cadmium (Cd), and copper (Cu) have been detected in worker bees and products of the hive such as honeycomb (Di et al. 2016). Daily sublethal exposure to these pollutants can alter immune and cognitive functions (Monchanin et al. 2021). In accordance with the above, combined exposure to pesticides and metals has also been found to affect detoxification and immune system functions, potentially increasing susceptibility to pathogens and disease (Goulson et al. 2015). Copper is a trace element, and copper-based compounds are among the most widely used active substances in plant protection products. Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), for instance, has been used for centuries to treat fungal and bacterial plant diseases (Lamichhane et al. 2018).

The Bordeaux mixture (copper sulfate plus lime) was developed at the end of the nineteenth century and is among the earliest and most successful chemical strategies for controlling downy mildew in vineyards (Lamichhane et al. 2018). Copper based plant protection products are still used extensively in organic as well as conventional agriculture

(Lamichhane et al. 2018). Meanwhile, an excessive amount of copper application may result in damage to soil biotic properties, and the restriction on copper fungicides has begun in some regions such as the European Union (Lamichhane et al. 2018). Copper-containing fungicides are widely used because of their broad-spectrum activity and multiple modes of action, which reduce the likelihood of resistance development. Furthermore, due to their moderate acute mammalian toxicity, they have historically been considered comparatively low-risk pesticides (Lamichhane et al. 2018). Nevertheless, the actual effects of copper exposure on honey bee health are not fully understood. Although required in trace amounts for physiological processes, copper becomes toxic when homeostatic regulation is exceeded (Di et al. 2016).

The present study aimed to evaluate the 24-hour acute oral toxicity of copper sulfate pentahydrate in worker honey bees under controlled laboratory conditions. In a 10-day chronic laboratory feeding bioassay with copper sulfate, the LC_{50} was estimated at approximately 66 mg/L, with worker mortality increasing markedly with increasing copper concentration, indicating strong toxicity under prolonged exposure conditions (Glavan et al. 2024). Likewise, honey bees have been shown to suffer from impaired learning and memory following exposure to environmental metal pollutants (Monchanin et al. 2021). In comparison, the US Environmental Protection Agency (EPA) has categorized copper compounds as "practically nontoxic" to honey bees with an acute oral LD_{50} > 100 μg/bee (Boone et al. 2012). While this classification indicates low acute hazard, it does not rule out subtler or longer-term effects that may warrant further study.

Copper-based compounds are generally considered to pose relatively low acute risk to honey bees when applied alone. However, recent evidence suggests that their combination with other pesticides, such as dinotefuran and triflumizole, may lead to additive or synergistic toxicity and increase mortality risk in honey bees (Jang et al. 2025). Although copper sulfate pentahydrate is frequently used in Türkiye and worldwide, there are few studies describing honey bee LC_{50} to copper sulfate pentahydrate. It is evident that a significant gap exists within the current discourse on bee safety and the assessment of pesticide hazards. The objective of this research was to test copper sulfate pentahydrate for acute toxicity in worker honey bees under laboratory conditions and to establish the median lethal concentration (LC_{50}) for the species. For metals like copper, acute toxicity generally requires elevated concentrations, while chronic exposure to low doses can cause considerable biochemical and physiological damage (Even et al. 2012). The 24-h acute LC_{50} value of copper obtained in this study reflects only the lethal effects of short-term, high-dose exposure and does not capture the sublethal effects (e.g., behavioral changes, immune suppression, learning/memory impairment) resulting from long-term exposure to low copper concentrations (Johnson 2015). Therefore, a comprehensive risk assessment of copper to honey bees must integrate acute toxicity data with chronic toxicity and sublethal effect studies. The important methodological distinction is that LC_{50} describes the concentration of a bioactive agent in diet or environment (ppm, mg/L) while LD_{50} denotes an absolute amount administered per individual (μg/bee), making it more appropriate for dietary exposure studies as ours where no exact quantity of consumption at the individual level can be controlled and trials remain analogous when considering LC.

Copper-based fungicides are widely used in agriculture; however, copper concentrations in floral resources, such as nectar and pollen, are generally reported at relatively low levels (Di et al. 2016). These concentrations are considerably lower than the 24-h acute oral LC_{50} value determined in the present study (4711 ppm). This suggests that acute copper intoxication of honey bees under field conditions may be unlikely when copper is encountered alone. Nevertheless, the environmental persistence of copper, its potential sublethal effects, and possible synergistic interactions with other pesticides (Jang et al. 2025) indicate that long-term risks warrant further evaluation through chronic toxicity studies. Furthermore, exposure to sublethal doses of widely used pesticides, such as the neonicotinoid thiamethoxam, has been shown to significantly disrupt crucial cognitive functions like associative learning, flower fidelity, and foraging behavior in honey bees (Çakmak et al. 2018).

MATERIALS AND METHODS

Honey Bees and Test Substance

Newly emerged (0-day-old) worker honey bees (*Apis mellifera*) were collected from a single healthy colony to minimize genetic variability among experimental individuals. Experimental units consisted of plastic observation cages, each containing 10 bees each. Cages were maintained in a controlled environmental chamber at 25 ± 2 °C and 65% relative humidity. Throughout the experiment, bees were provided with ad libitum access to 50% (w/v) sucrose solution. Copper sulfate pentahydrate (CAS No. 7758-99-8) was used as the test substance. For the oral toxicity assay, stock solutions were prepared by dissolving $CuSO_4 \cdot 5H_2O$ in 50% sucrose. All test solutions were freshly prepared on the day of exposure and kept in the dark until use.

Experimental Design and Exposure Procedure

Prior to the definitive assay, a preliminary range-finding test was conducted to identify an appropriate concentration interval for LC_{50} estimation. Bees were exposed to 50, 100, 250, 500, 1000, 2500, 5000, 7500, and 10,000 ppm, and mortality was recorded at 24 h. Based on the observed mortality range of 0–100% (approximately 4500–7000 ppm), eight concentrations (4500, 5000, 5250, 5500, 5750, 6000, 6500, and 7000 ppm) were selected for the definitive test to allow robust modeling of the concentration–mortality relationship. For each concentration and the control group, three replicate cages were used, a total of 27 cages ((8 concentrations + 1 control group) × 3 replicates). The negative control group received only 50% sucrose solution without $CuSO_4 \cdot 5H_2O$. At the start of the experiment, bees were food-starved prior to exposure to promote feeding.

Test solutions were then provided ad libitum via micro feeders. Bee status was checked hourly throughout the 24-h exposure period. Mortality was defined as the absence of any antennal movement upon gentle stimulation with forceps. Cumulative mortality was recorded, with particular attention to the 4h and 24h time points. For assay validity, mortality in the negative control was required to remain below 10%.

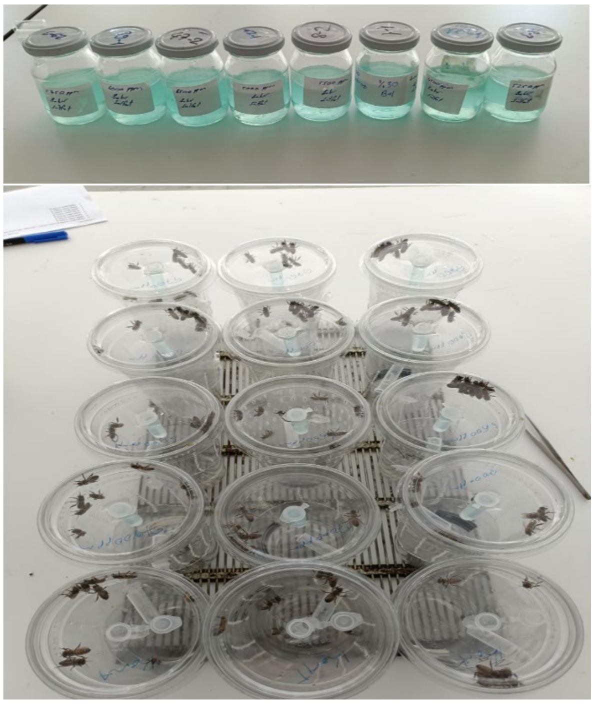

Figure 1. Laboratory cage bioassay setup used in the acute oral toxicity test, showing newly emerged worker honey bees confined in plastic observation cages and fed ad libitum with sucrose solutions freshly prepared with copper sulfate pentahydrate

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics (version 29.0 Mortality data were analysed using probit regression, and model parameters (slope and intercept) were estimated by maximum likelihood. Using the fitted model, the concentrations predicted to cause 5% (LC_5), 10% (LC_{10}), 20% (LC_{20}), 50% (LC_{50}), and 90% (LC_{90}) mortality, along with their 95% confidence intervals, were calculated. The dose–response relationship predicted by the probit model is shown in Figure-2. Model goodness-of-fit was evaluated using the Pearson chi-square (χ^2) statistic. All analyses were based on cumulative mortality measured at the end of the 24h exposure period.

RESULTS

A clear, concentration-dependent increase in predicted acute oral mortality of copper (Cu) was observed across the tested concentration range, as shown in the concentration–response curve (Figure-2). LC estimates from the model and their 95% confidence intervals (CIs) are presented in Table 1. The selected exposure range (4500–7000 ppm) effectively spanned the LC_{50} region, allowing precise discrimination of mortality responses within 24 h. The 24h LC_{50} determined from the probit analysis was 4711 ppm, CI:4309-4952. The relatively narrow confidence interval indicates that the precision of estimation is higher near the point of inflection in the response curve. LC_5 was 3006 ppm (95% CI: 1794–3620), at lower effect values, whereas the estimates for LC_{10} and LC_{20} were 3383 ppm (95% CI: 2357–3907) and 3839 ppm (95% CI: 3036–4257), respectively.

As expected, lower-effect concentrations showed greater variability, while higher-effect concentrations provided more precise estimates. Control mortality remained 0% (0/30), confirming assay validity. From a probit regression perspective, $CuSO_4 \cdot 5H_2O$ caused lethal effects in worker honey bees (*A. mellifera*) at ppm concentrations within 24 h of exposure. Results from this study (LC_{50} = 4711 ppm; LC_{90} = 6040 ppm) provide quantitative anchor points to inform the acute risk characterization of copper-containing plant protection products (Figure 1), as well as a discussion of practical exposure thresholds and comparability across future studies.

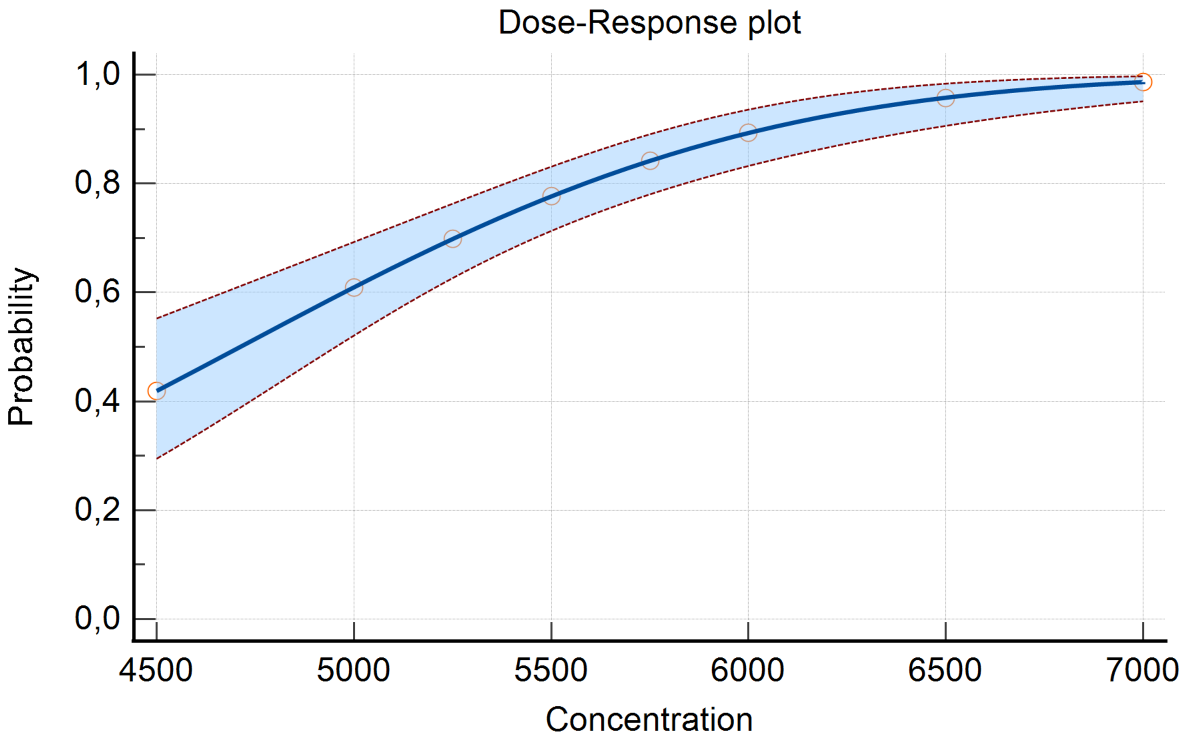

Figure 2. Dose response relationship (24 h) for *Apis mellifera* worker bees exposed to copper sulfate pentahydrate. The solid line represents the probit model estimate of mortality probability as a function of concentration (ppm, log scale). The shaded area indicates the 95% confidence limits. LC_5, LC_{10}, LC_{20}, LC_{50}, and LC_{90} point estimates are shown with horizontal error bars.

LC_5, LC_{10}, LC_{20}, LC_{50}, and LC_{90} represent the model-predicted concentrations (ppm) expected to cause 5%, 10%, 20%, 50%, and 90% mortality at 24 h, respectively. The "Estimate" column reports point estimates from the probit model, whereas "Lower CI" and "Upper CI" report the corresponding 95% confidence limits. The wider confidence intervals at lower effect levels (LC_5–LC_{20}) indicate greater uncertainty in this region, while estimation is relatively more precise around LC_{50}. The estimated LC values and their corresponding confidence intervals are presented in Table-1.

Table-1. LC threshold values and 95% confidence intervals for copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) in newly emerged (0-day-old) worker honey bees (*Apis mellifera*) after 24h acute oral exposure (probit analysis).

LC Level	**Estimate (ppm)**	**Lower CI (ppm)**	**Upper CI (ppm)**
LC_5	3006	1794	3620
LC_{10}	3383	2357	3907
LC_{20}	3839	3036	4257
LC_{50}	4711	4309	4952
LC_{90}	6040	5791	6465

The 24h acute oral toxicity of copper sulfate pentahydrate ($CuSO_45H_2O$) to 0-day-old worker honey bees (*Apis mellifera*) was quantified and the LC_{50} (24 h) was estimated to be 4711 ppm in a probit regression model. The reporting of additional percentiles (LC_5, LC_{10}, LC_{20} and LC_{90}) improves the characterization of risk by taking into account uncertainty across regions with both low and high mortality instead of making conclusions based on a single mid-point estimate only (Finney 1952). The increasing trend in mortality with concentration is also consistent with the general dose–response profile observed for metals (Mogren & Trumble 2010) and corresponds to previous work linking heavy metal exposure to concentration-dependent increases in honey bee mortality (Di et al. 2016).

DISCUSSION

Interpreting the acute LC_{50} estimate requires consideration of the fact that copper has been examined more often in chronic or sublethal contexts in the literature (Johnson 2015). Glavan et al. (2024) reported LC_{50} = 66.7 mg/L for $CuSO_4$ following 10 days of chronic oral exposure in *Apis mellifera carnica* workers. In dilute aqueous solutions, mg/L and ppm are often used interchangeably as approximations in toxicological studies. Viewed in this context, a substantially lower chronic LC_{50} relative to the acute LC_{50} is consistent with general toxicological expectations that toxicity may increase with longer exposure through time-dependent accumulation and physiological costs (Even et al. 2012). Related work also emphasizes that prolonged exposure to metals can increase detoxification demands, oxidative stress, and metabolic strain (Mogren & Trumble 2010). However, the discrepancies observed in these studies cannot be attributed solely to exposure duration, as comparability is affected by the biological material and experimental conditions. Glavan et al. (2024) used adult workers collected within the colony in summer, whereas we used newly emerged (0-day-old) workers.

There is an age-dependent sensitivity to chemicals that play a role in bee toxicology, and tolerance to toxicants can vary significantly among age classes (Johnson 2015). In some instances, foragers have been found to be even more sensitive than in-hive workers to systemic toxicants (Tosi & Nieh 2019). The feeding matrix is also important: Glavan et al. (2024) used 1.5 M sucrose, whereas 50% (w/v) sucrose solution was utilized in the protocol described here. Furthermore, cage environment, feeder designs, and ad libitum feeding can influence intake and, therefore, the amount consumed due to oral dosing. Thus, acute LC comparisons between protocols should be cautiously evaluated based on standardized procedures used in each study (Medrzycki et al. 2013). Copper is an essential trace element, but at elevated concentrations it can induce oxidative stress via increased reactive oxygen species, potentially impairing enzymatic activity and cellular membrane integrity (Even et al. 2012, Johnson 2015).

Copper salts have been shown to influence gut phosphatase activity in honey bees and the effects may be related to digestive and metabolic processes (Bounias et al. 1996). There is also additional evidence indicating that metal pollutants have negative, cumulative effects on cognitive function (Monchanin et al. 2021). Thus, in addition to mortality thresholds, it would be useful to consider both sublethal impairment and its consequences for colony performance as a further dimension of risk (Johnson 2015). The behavior aspect of copper exposure should also be considered. In two-choice feeding assays, Glavan et al. (2024) observed avoidance behavior at even higher copper levels. In line with that, acute or sublethal heavy metal exposure was reported to alter feeding behavior. However, for some toxic elements, bees may maintain intake at levels that are lethal (Burden et al. 2016). Lastly, what the acute LC_{50} obtained here means under environmentally relevant exposure conditions is still an open question.

Metal exposure to honeybees occurs mainly via nectar, pollen and water (Nagajyoti et al. 2010). Importantly, copper is rarely found in isolation in agricultural settings; a recent study by Jang et al. (2025) demonstrated that copper hydroxide can interact synergistically with insecticides such as dinotefuran, posing a significant interactive risk to honey bees in field settings. Metals can also accumulate in bee tissues and hive products, supporting the use of honey bees as bioindicators of environmental contamination (Leita et al. 1996). Field studies have reported metal contamination of bees and bee products (Giglio et al. 2017). Furthermore, hyperaccumulator plants can elevate local exposure in certain areas (Balafrej et al. 2020).

In conclusion, this study quantifies the 24h acute oral toxicity threshold of $CuSO_4{\cdot}5H_2O$ in worker honey bees and provides a more detailed framework for risk assessment based on thresholds from LC_5 to LC_{90} (Finney 1952). Furthermore, as clearly shown in the dose-response curve (Figure-2), the sharp increase in mortality at higher concentrations and the wider confidence intervals at lower effect levels (LC5-LC20) highlight the need to consider the entire toxicity profile rather than relying solely on a single LC50 value for environmental risk assessments. Along with the chronic LC_{50} reported by Glavan et al. (2024), the results here suggest that copper toxicity is greatly influenced by exposure period and experimental context. Because of the widespread use of copper-based plant protection products, integrated investigations of acute, chronic, and sublethal effects are required to develop strategies to protect bee health (Johnson 2015).

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Data availability: Data are available from the corresponding author upon reasonable request.

Ethics approval: Not applicable / Not required for invertebrates (honey bees) under institutional guidelines.

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