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Adverse effects of entomopathogenic nematodes on honey bees following foliar application under laboratory conditions

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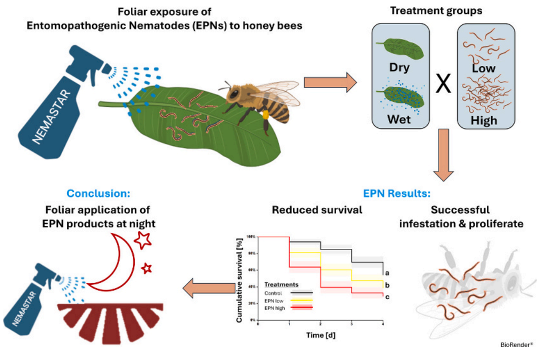
HIGHLIGHTS

- Foliar application of *Steinernema carpocapsae* reduced in a decrease in adult honey bee survival.
- Exposure under wet applications significantly increased mortality.
- Nematode infestation and proliferation confirmed in adult honeybees.
- EPN infestation and proliferation depended on dose and application method used.

GRAPHICAL ABSTRACT



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ABSTRACT

The need for sustainable crop protection has intensified interest in entomopathogenic nematodes (EPNs) as biological alternatives to synthetic agrochemicals. However, their potential non-target effects on pollinators remain poorly characterized, and regulatory approvals often rely on limited data. Here, we provide the first demonstration of adverse effects of foliar-applied *Steinernema carpocapsae* (Nemastar®) on adult honey bees, *Apis mellifera*. Using a modified regulatory protocol (USEPA 850.3030), adult honey bees and greater wax moth larvae (*Galleria mellonella;* positive control) were exposed for 96 h to field-realistic low and high EPN doses (0.25–2.5 × 10^6 infective juveniles m^2) under dry and wet application scenarios. Adult honey bee and greater wax moth larvae mortality was recorded daily. *Steinernema carpocapsae* significantly reduced survival in both species, with the highest mortality under the wet and high-dose conditions. Nematode infestation and proliferation were confirmed in deceased individuals using White traps and standardized counts, revealing species-, dose-, and application-dependent differences. Our results provide novel evidence that foliar surface application of *S. carpocapsae* can adversely affect adult honey bees. We recommend restricting foliar applications to nighttime (i.e., after dusk) to reduce the risk of exposure to non-target pollinators. We further emphasize the urgent need for standardized regulatory studies for EPN products to ensure sufficient evidence of environmental safety before approval and widespread use.

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1. Introduction

Agrochemicals play a decisive role in global biodiversity declines (Brühl and Zaller, 2019), with synthetic pesticides contributing to widespread losses of insect populations, including pollinators (Dicks et al., 2021). As pollinators provide critical ecosystem services and underpin agricultural productivity, reducing their exposure to harmful agrochemicals has become a priority for sustainable food production, driving the development of effective and environmentally compatible pest management alternatives. Biological control agents, including entomopathogenic nematodes (EPNs), are widely promoted as such alternatives due to their natural origin, relative host specificity, and compatibility with integrated pest management (IPM) programs (Gaugler, 2002).

Entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* are obligate insect parasites. Infective juveniles (IJs) enter hosts through natural openings and release symbiotic bacteria (*Xenorhabdus* spp. or *Photorhabdus* spp.), leading to host death via septicemia (Campos-Herrera, 2015; San-Blas et al., 2024). Within the host cadaver, the IJs reproduce, and the resulting offspring (i.e., new IJs) disperse from their original host in search of new hosts. Their ease of mass production, persistence in soil, and efficacy against soil-dwelling insect pests have made them valuable tools in agricultural pest management (Lacey and Georgis, 2012; Gümüş Askar et al., 2022; Blanco-Pérez et al., 2024). Although traditionally applied as soil drenches (Shapiro-Ilan and Dolinski, 2015), EPNs are increasingly formulated for foliar sprays targeting above-ground pests, including the tomato leafminer (*Tuta absoluta*) and onion thrips (*Thrips tabaci*) (Al-kazafy et al., 2016; Metwally et al., 2025). Foliar applications require high moisture, which enhances IJ survival, mobility, and adherence to insect cuticles, increasing infection success (Grant and Villani, 2003; Radová and Trnková, 2010; Rohde et al., 2010; Shapiro-Ilan et al., 2006; Shapiro-Ilan and Lewis, 2024). These conditions may simultaneously increase exposure risk for non-target insects, such as pollinators present on treated vegetation or during application, yet data on these risks remain scarce.

Regulatory authorities often classify EPNs as minimal-risk products, allowing expedited approval with limited or no requirement for pollinator safety data (Hokkanen et al., 2003; Ramakuwela et al., 2025). However, infections in non-target organisms, including beneficial insects, have been reported (e.g., Bathon, 1996; Sandhi and Reddy, 2019; Erler et al., 2022). For instance, laboratory studies using *Heterorhabditis* spp. and *Steinernema* spp. revealed a dose-dependent increase in mortality in adult honey bees and their brood (Hackett and Poinar, 1973; Shamseldean et al., 2004). Furthermore, Dutka et al. (2015) demonstrated that bumble bees exposed to EPNs via contaminated soil exhibited high infestation and mortality rates, questioning the safety of EPN products. Despite growing interest and demand for foliar EPN applications (e.g., Sáenz-Aponte et al., 2020; Moisan et al., 2024; Perier et al., 2025), their potential risks to non-target species, particularly pollinators, require further evaluation.

This study investigated the effects of foliar surface applications of *Steinernema carpocapsae* IJs on adult honey bee (*Apis mellifera*) survival under laboratory conditions, comparing direct overspray (wet) and post-overspray (dry) scenarios. We assessed (i) whether *S. carpocapsae* IJs can infect and proliferate within adult honey bees following foliar exposure, and (ii) how dose and application scenario influence infection success and mortality. Based on past studies (e.g., Rohde et al., 2010; Dutka et al., 2015), we hypothesized that *S. carpocapsae* exposure under high-humidity conditions (direct overspray) would significantly enhance infestation success, resulting in increased proliferation rates and elevated host mortality. Our findings provide empirical data to inform policymakers and highlight the need for standardized pollinator risk assessments for future EPN products prior to market authorization.

2. Methods

2.1. Set-up

The study was conducted at the Institute of Bee Health and Swiss Bee Research Centre Agroscope, Bern, Switzerland, between April and August 2022. To investigate the impact of *Steinernema carpocapsae* IJs on adult female (worker) honey bees (*A. mellifera*) via overspray (i.e., wet) and foliage exposure to dried IJ suspension residue, we followed a modified version of the OCSPP 850.3030 foliage protocol (USEPA, 1996). In brief, workers were obtained from three local, non-related, queenright colonies managed using Best Management Practices including *Varroa destructor* mite treatments in the previous summer and winter (i.e., formic acid 70% and oxalic acid 2.7%, respectively) (Dietemann et al., 2013). To obtain worker honey bees of a known age cohort, we selected brood frames containing individuals that were within 24 h of emergence by uncapping sealed brood cells and inspecting adult coloration (Human et al., 2013). Brood frames were then transferred to a laboratory incubator set to 34.5 °C, 60% relative humidity, and complete darkness for a period of 24 h (Williams et al., 2013). Upon emergence, workers were examined for disease symptoms, physical abnormalities, or parasitic mite (*V. destructor*) infestation (Dietemann et al., 2013). Individuals with abnormalities (e.g., deformed wings) or *V. destructor* infestation were excluded to avoid confounding factors. In addition to the honey bee workers, L4 larvae of the greater wax moth (*Galleria mellonella* L.) were purchased from a local supplier (Fischereibedarf N. Wenger AG, Bern, Switzerland). We used *G. mellonella* as a positive control in our study as they are commonly used in mass production of EPNs including *S. carpocapsae* (Metwally et al., 2012). Newly emerged bees from all three colonies as well as the greater wax moth larvae were randomly allocated to plastic hoarding cages [750 cm^3] that contained a precut rectangular piece [10 × 15 cm] of a true banana leaf (*Musa tropicana*) and a mesh grid on the lid to enable ventilation (see Supplementary Materials (SM) Fig. 1A). To ensure leaves were kept fresh and did not welk during the exposure period, damp filter paper was placed beneath the leaves. Cages consisted of either ten honey bee workers ($N_{\text{total cages}} = 54$; $N_{\text{total bees}} = 540$) or ten greater wax moth larvae (except for one cage only having six larvae; $N_{\text{total}} = 506$). In the case of the honey bees, each cage was equipped with a 5 mL syringe (Codan Medical AG, Switzerland) supplying ad libitum sucrose solution (50% [*w*/*v*]) and a pollen feeder containing honey bee–collected corbicular pollen (90%) mixed with honey (10%) during the exposure period.

2.2. Treatment exposure and preparation of IJ suspensions

The experimental design consisted of three experimental groups: (i) test substance (EPN treatment), (ii) positive control (i.e., dimethoate) and, (iii) a negative control (i.e., tap water). For each treatment group two exposure scenarios were assessed (i.e., direct overspray on leaf (wet scenario) and dry residues on leaf (dry scenario)). Although not required by the guideline (OCSPP 850.3030), dimethoate was included as a positive control to confirm assay reliability and sensitivity, consistent with its widespread use in regulatory toxicity testing (Gough et al., 1994). The EPN treatment included two field-realistic surface application rates of *S. carpocapsae* IJs: a low dose (i.e. 250,000 IJs m^{-2}) and a high dose (i.e. 500,000 IJs m^{-2}). While the product used in this study is currently not approved for foliar surface application in Switzerland, the chosen applied concentrations were based on the recommended application rates of a comparable product (Capsanem®, Koppert, n.d.). This resulted in a total of eight experimental treatment groups: 1. No IJs and dry scenario (= negative control dry), 2. No IJs and wet scenario (= negative control wet), 3. Dimethoate and dry scenario (= positive control dry), 4. Dimethoate and wet scenario (= positive control wet), 5. IJs low dose and dry scenario (= EPN low dry), 6. IJs low dose and wet scenario (= EPN low wet), 7. IJs high dose and dry scenario (= EPN high dry), and 8. IJs high dose and wet scenario (= EPN high wet). As both honey bee workers and the wax moth larvae were exposed to these eight treatments, the fully crossed experimental design had a total of 16 treatment groups. All treatment IJ suspensions were made using tap water according to standardized guidelines (OECD, 1998). The EPN product (Nemastar®) was purchased online via www.bioprotect.ch from Agroline Bioprotect (Aesch, Basel, Switzerland). The product consisted of approximately 50 million *S. carpocapsae* IJs which were mixed with 500 mL of water to obtain a stock IJ suspension with a concentration of 100,000 IJs mL^{-1}. The concentrations were confirmed by using a 5 μL aliquot of each IJ suspension and the nematode density was determined by taking the average of three counts using a Neubauer counting chamber (Thermo Fischer Scientific, USA) under light microscopy (Olympus BX41, Switzerland). The positive control, dimethoate (Sigma-Aldrich, USA, Lot BCCF3993, CAS: 60–51-5; Stähler Suisse SA, Zofingen, Switzerland), was produced by mixing 30.1 mg of dimethoate in 1 L of tap water to obtain a test solution with a concentration of 0.031 μg μL^{-1} active ingredient (a.i.). The surface application rate chosen was 4550 μg m^{-2} corresponding to a field-realistic rate of 45.5 g ha^{-1} (Pistorius and Steeger, 2025). The control solution consisted of only tap water.

To simulate a realistic 2D application in the field at a maximum water volume of 1250 L ha^{-1}, a square of 1 m^2 was marked out for the overspray application of the test units, which were placed within the designated area. To verify that spraying and nozzle passage (Adjustable brass nozzle (Super Star 1.25, Spray-Matic 1.25 P/N), Birchmeier Sprühtechnik AG, Stetten (Aargau), Switzerland) did not affect EPN viability or physical integrity, IJs were examined microscopically before and immediately after passage through the handheld sprayer nozzle. No visible damage or loss of viability was observed between pre- and post-application samples. IJ integrity was also confirmed following spraying onto foliage. Representative micrographs are provided in the Supplementary Information (SM Fig. 2), indicating that nozzle-related effects on IJ viability were negligible under the application conditions used.

The water output of the hand-held sprayer (Super Star 1.25 / 360°, Birchmeier Sprühtechnik AG, Stetten (Aargau), Switzerland) was determined in advance and revealed a water output of 125 mL per 17 s. Based on the sprayers target water output volume (125 mL m^{-2}), 1 L of each application solutions for each treatment group were prepared accordingly. Then, the treatment IJ suspension was evenly sprayed for 17 s over the designated 1 m^2 area consisting of the test units assigned to one of the four treatment suspensions (e.g., negative control, positive control, EPN low, and EPN high). After each application the remaining volume was measured, to calculate the definitive treatment dose per square meter (see SM Table 1). Prior to being randomly assigned to one of the eight treatment groups, all insect specimens were anaesthetized with CO_2 for 10 s. For the wet exposure scenario, specimens were added into the hoarding cage and then the treatment IJ suspension was sprayed directly over the designated 1 m^2 onto the leaves and specimen. For the dry exposure scenario, hoarding cages were sprayed, left to dry for 60 min at RT and indirect natural light - at which point the leaves were completely dry and the specimen were added. The study consisted of three independent experimental runs conducted on different days, each using newly prepared insect specimens and treatment IJ suspensions to ensure full biological and technical replication. Within each run, each treatment group comprised three hoarding cages. All hoarding cages were kept in an incubator at 25 °C and 60% RH) and complete darkness (Williams et al., 2013).

2.3. IJ survival on foliage

As a preliminary, independent assessment, IJ survival on treated foliage was evaluated to confirm nematode viability under the experimental conditions at the time of insect introduction (dry scenario). Three additional cages exposed to the low-dose IJ treatment suspension were prepared without insects. This assessment was conducted under dry conditions following a 60-min drying period at RT, representing a conservative ('worst-case') scenario as described above, as EPN survival is known to be higher under wet conditions (Ramakrishnan et al., 2022). After drying, cages were examined under a stereomicroscope (Olympus, SZX7, 20× magnification). Eight randomly selected visual fields per cage were assessed, recording total IJs and immobile (dead) individuals to calculate mortality. Counts were repeated immediately after the 60-min drying period (i.e., time point 0) and at 5 min intervals until all IJs were deceased. The IJs were classified as dead if no movement was observed following gentle agitation.

2.4. Adult honey bee and greater wax moth larvae survival, IJ infestation and IJ proliferation

Survival was recorded daily for four days post exposure, consistent with the acute toxicity framework of the OCSPP 850.3030 foliage protocol (USEPA, 1996), although EPN-induced mortality may extend beyond this period (Dillman et al., 2012). Irrespective of the treatment group, the carcasses of deceased specimen were transferred to White traps (White, 1927), to facilitate the collection of nematodes proliferating within the infected carcasses. The White traps were designed according to the protocol of Orozco et al. (2014). In brief, each trap consisted of two petri-dishes of different sizes (i.e., 30 mm and 60 mm diameter). The smaller dish was placed inside the larger dish and a circular filter paper [25 cm^2] was placed in the smaller dish, upon which the deceased specimen was placed. Then, 20 mL of tap water was added to the larger dish, and the lid of the larger petri-dish was used to cover the petri-dishes. Given the insect specimen is infested, the IJs will eventually emerge from the carcasses and migrate away from the small petri-dish in search of a new host and eventually fall into the water from the larger petri-dish (SM Fig. 1B). The White traps were maintained in an incubator and kept in complete darkness at 24 °C and 60% RH. Specimen was left within the white traps for at least three weeks to ensure that IJs had sufficient time to proliferate and migrate from the carcasses. Following Dutka et al. (2015), IJ counts were performed to assess the average number of IJ in three aliquots of 50 μL of water from each White trap, with the counts then being adjusted to estimate the total number of nematodes produced by each infected bee based on the total volume of water remaining in the White trap. Resulting in the following equation:

$$Total\ Infective\ Juveniles = \frac{remaining\ liquid\ (\mu L)*mean\ number\ of\ nematodes\ per\ aliquot}{volume\ aliquot\ (50\ \mu L)}$$

If no IJs were detected in the White trap water solution, the carcasses were dissected under a binocular (Olympus SZX7, Evident Europe GmbH, Hamburg, Germany) to confirm the absence of IJ. In addition, all individuals that survived the four-day exposure period were dissected under the binocular to confirm their infestation status (SM Fig. 1C&D).

2.5. Statistical analysis

Statistical analyses were performed using STATA16 (StataCorp. 2019. *Stata Statistical Software: Release 16*. College Station, TX:

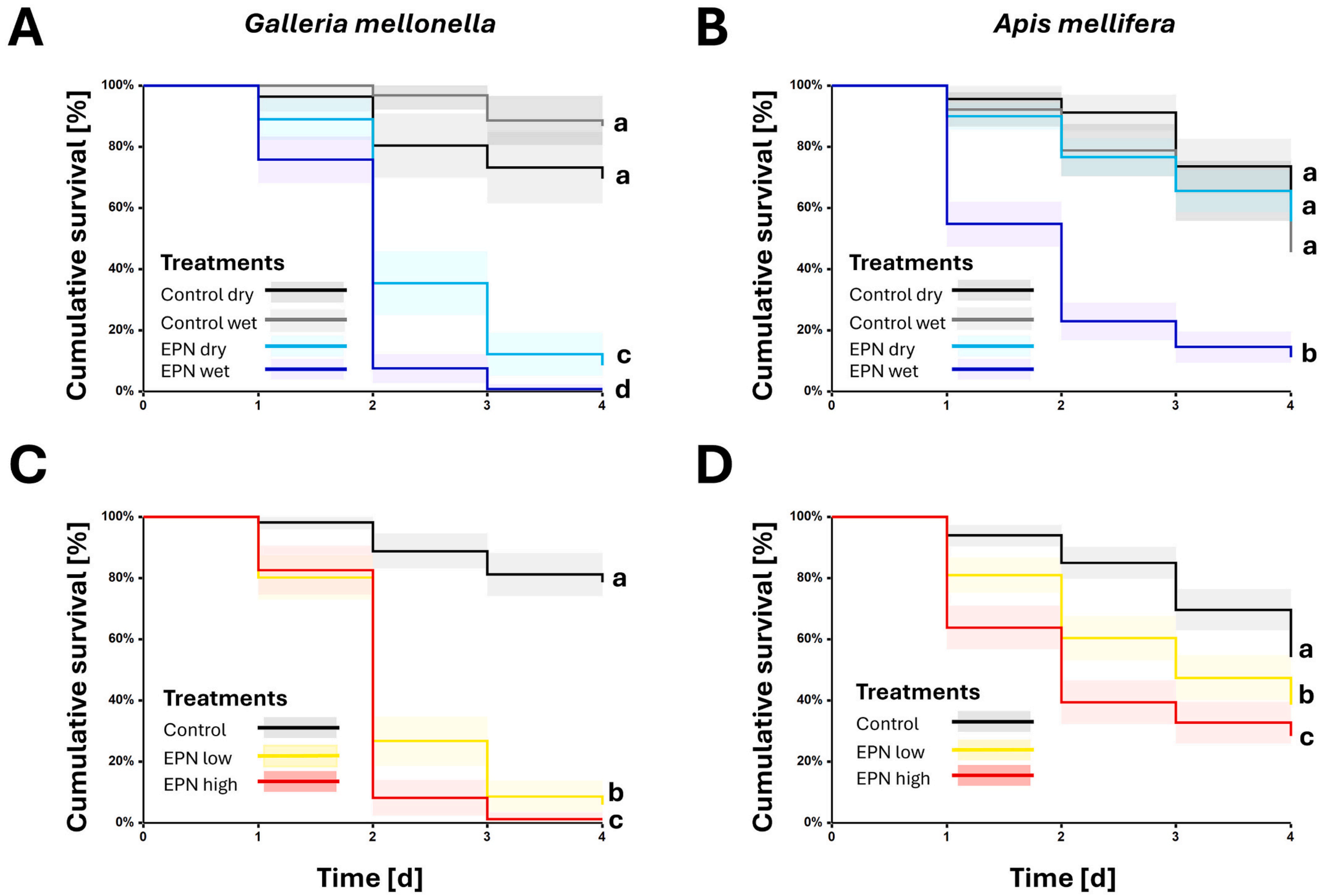

Fig. 1. Cumulative survival rates visualized using Kaplan-Meier survival curves. Using two application scenarios (i.e., dry and wet) the effects of exposure to varying doses (i.e., low and high) of an entomopathogenic nematode (EPN) were assess on the survival of (**A & C**) the greater wax moth larvae (*Galleria mellonella*) and (**B & D**) worker honey bees (*Apis mellifera*). With the exception dry exposure to *G. mellonella*, irrespective of the species, the wet exposure as well as the exposure to EPNs at both the low and high dose had a significant negative effect on survival rates (all P's < 0.01). While the lines represent different treatment groups, the shaded areas around the lines represent the 95% confidence intervals. Significant statistical differences amongst treatments ($P < 0.05$) were identified using Bonferroni-corrected post-hoc pairwise comparisons and are denoted by lowercase letters (i.e., a, b, c, d).

StataCorp LLC), whereas all statistical figures were created using NCSS23 (NCSS version 23, Statistical Analysis Software, Kaysville, Utah, USA). All outcome variables (i.e., mortality [%], infestation rates [%], nematode counts [n]) were visually inspected using quantile-quantile plots as well as being tested for normality using the Shapiro–Wilk's test ($P < 0.05$) and the Levene's test for homogeneity of variances. Associations between explanatory and response variables were analysed using generalized linear or logistic mixed-effects models, as appropriate. Models were fit using the functions *glm* or *ologit*, respectively. Individual specimen (i.e., adult bees or greater wax moth larvae) were treated as independent observations, while 'exposure' (i.e., IJ exposed or non-exposed), 'species' (i.e., *A. mellifera* and *G. mellonella*), 'exposure scenario' (i.e., dry or wet) and 'dose' (i.e., low or high) were included as fixed explanatory factors in all models. Interaction terms (e.g., '*exposure scenario#dose*') were incorporated as fixed factors where relevant. To account for nesting, 'run' (i.e., replicate) was included as a acovariate and 'cage' was included as a random effect to avoid pseudo replication by accounting for individuals nested within cages. For each multiple regression model, a stepwise backward elimination approach was applied to determine the mode of best fit (Wiegand, 2010). Best fit models were chosen by comparing every multilevel model with its single-level model counterpart using both a likelihood ration (LR) test as well as the Akaike information criterion (AIC) using the function *lrtest* and *estat ic*, respectively. Post-hoc comparisons amongst treatment groups for all variables were conducted using pairwise Bonferroni multiple comparisons test (*bmct*), using the function *mcompare(*Treatments) and option *bonferroni*, whenever necessary. Specific details to each model, including Shapiro-Wilk's results, applied distributions, covariates, fixed effects, as well as the applied STATA functions for each variable can be found in the SM Table 2.

IJ survival at 0, 5 and 10 min was compared using a Kruskal-Wallis one way ANOVA on ranks (corrected for ties), followed by Bonferroni-corrected all pairs multiple comparison tests. The remaining survival analyses were performed using the *mestreg* function for multilevel survival models (Cleves, 2002) and survival time was set using the function *stset* and the *if* option was used for censored individuals. Differences in survival amongst the different explanatory factors were fitted using the *streg* function for multi-level survival models (Leckie, 2010). Survival was calculated by using cumulative survival [%] after four days for each treatment and Kaplan Meier curves with 95% confidence intervals (CI) were used to visually display the data. The variable infestation rate [%] was assessed using logistic regression, whereby the conditional distribution of the regression was assumed to be Bernoulli. IJ count [n] was modelled using a Gaussian distribution, where the fixed explanatory variables kept in the model were 'species', 'exposure scenario' and 'dose' as well as including 'run' as a random effect. Whenever appropriate, the means ± standard error (SE), adjusted for distribution, are reported in the text. For each tested variable summary statistics are provided in SM Table 3, including sample size, mean, standard error, and the upper and lower bounds of the 95% confidence interval.

3. Results

3.1. Survival

IJ survival declined significantly after drying (Kruskal-Wallis; $\chi^2 = 61.73$, df = 2, $P < 0.001$), decreasing from 100% ± 0 at 0 min to 8.2% ± 26.45 at 5 min and 0% ± 0 at 10 min (median [%] ± SD). Post-hoc Bonferroni comparisons confirmed significant differences amongst all time points (P's < 0.001; SM Fig. 3). Dimethoate exposure significantly increased insect specimen mortality compared with controls ($z = -17.04$; $P < 0.001$). In contrast, the control exposure scenario (i.e., dry or wet) revealed no significant effect ($z = 1.25$; $P < 0.21$). Overall control mortality after four days was 45%, whereas dimethoate caused 100% mortality within one day (SM Fig. 4).

Across both species, the EPN-related explanatory variables (i.e., exposure, exposure scenario, and dose) significantly affected survival rates (all z's < 11.0; all P's < 0.001; SM Table 2). Furthermore, a significant species-specific difference in survival rates was observed between *G. mellonella* and *A. mellifera* independently of treatment ($z = -5.12$; $P < 0.001$; SM Table 2). Subsequently, to facilitate the overview of the effects of the explanatory variables, we ran separate models for the species as well as independent models assessing the effect of exposure scenario as well as dose.

For both species, control survival was not significantly affected by the exposure scenario (i.e., dry or wet; both z's > − 2.39; both P's > 0.10), with cumulative survival of 78 ± 5% for *G. mellonella* and 54 ± 1% for *A. mellifera*, respectively (cumulative survival [%] ± S.E.; Fig. 1A&B). However, wet EPN exposure significantly increased mortality in both species (both z's > − 2.12; both P's < 0.034; Fig. 1A&B). While both EPN dry and wet treatment groups for *G. mellonella* significantly differed from their respective controls (all P's < 0.001; Fig. 1A), the contrary was the case for *A. mellifera*. Here, only the EPN wet treatment group significantly differed from the controls (both $P < 0.001$; Fig. 1B). For *A. mellifera*, the EPN dry treatment group did not significantly differ from either control treatment group (both P's > 0.20). Lastly, for both species, the EPN wet treatment group showed the significantly highest mortality rates (all z's < −8.49; all P's < 0.001; Fig. 1A&B), with survival rates being at 0% and 11.2 ± 2% for *G. mellonella* and *A. mellifera*, respectively. A significant dose-dependent decrease in survival was also detected in both species (both z's > 2.22; both P's < 0.02), with the high dose resulting in an increased mortality rate compared to the low dose (Fig. 1C&D). Overall, for both *G. mellonella* and *A. mellifera*, this resulted in control survival being the highest (78.6 ± 4%; 54.1 ± 4%), followed by the low EPN (6.1 ± 2%; 38.6 ± 4%) and the high EPN treatments (1.2 ± 1%; 28.3 ± 3%), respectively (cumulative survival [%] ± S.E.; Fig. 1C&D).

3.2. IJ infestation rates

All control groups were free of EPN infestation. The overall IJ infestation rates significantly differed between the species ($z = -5.74$; $P < 0.001$), where *G. mellonella* revealed higher infestation rates compared to *A. mellifera*. This effect was consistent when comparing the respective treatment groups to one another across the species (all z's < −5.74; all P's < 0.001). Irrespective of the species, the exposure scenario significantly affected the infestation rate ($z = 4.26$; $P < 0.001$), where

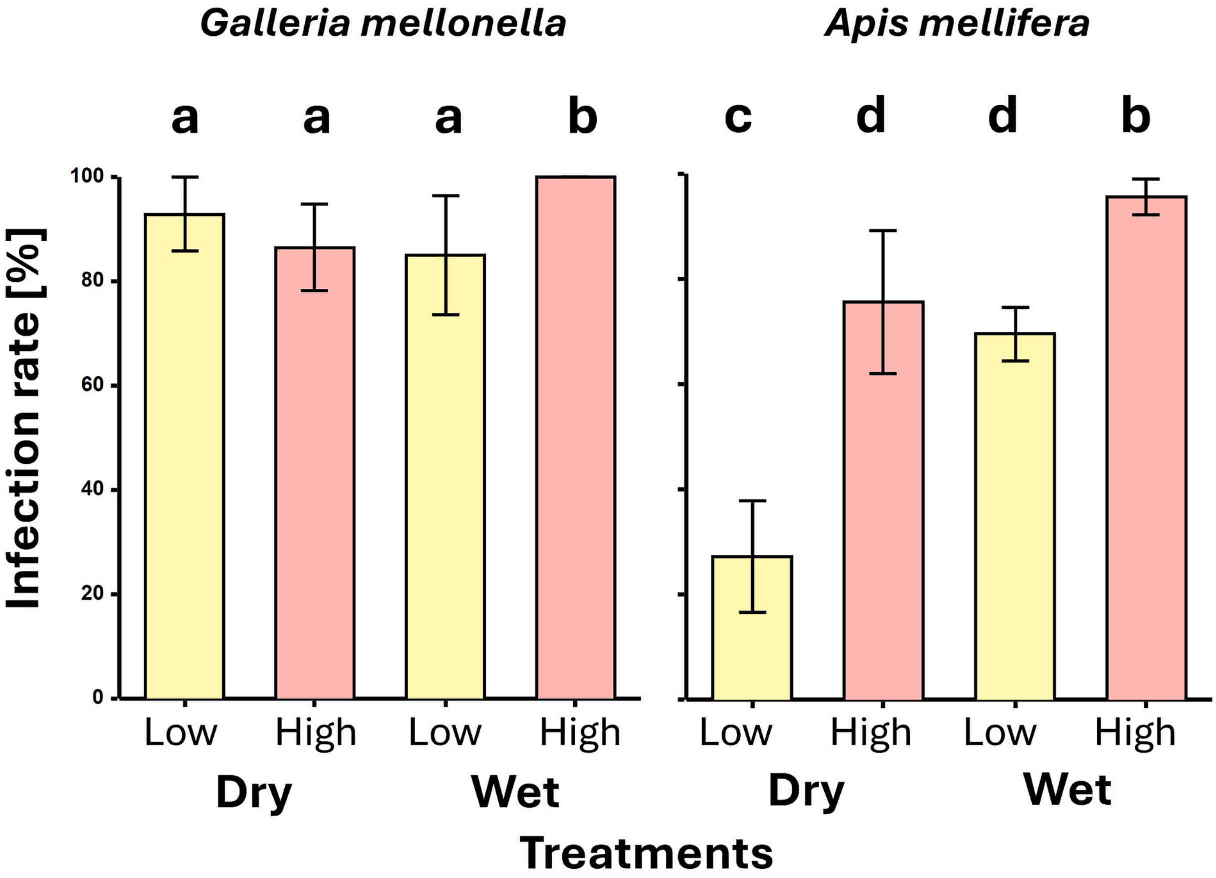

Fig. 2. Infestation rates of *Steinernema carpocapsae* under varying (i.e., dry or wet) foliar exposure scenarios. Error bar charts show infestation rates of greater wax moth larvae (*Galleria mellonella*) and worker honey bees (*Apis mellifera*) exposed to low and high concentrations of entomopathogenic nematodes (EPNs) under wet (direct overspray) or dry (post-overspray) conditions. Control treatments revealed no infestation and are therefore not displayed. Both the concentration as well as the exposure scenario significantly affect the infestation rates across both species (both P's < 0.001). Significant differences amongst treatments ($P < 0.05$) were identified using Bonferroni-corrected post-hoc pairwise comparisons and are denoted by lowercase letters (i.e., a, b, c, d).

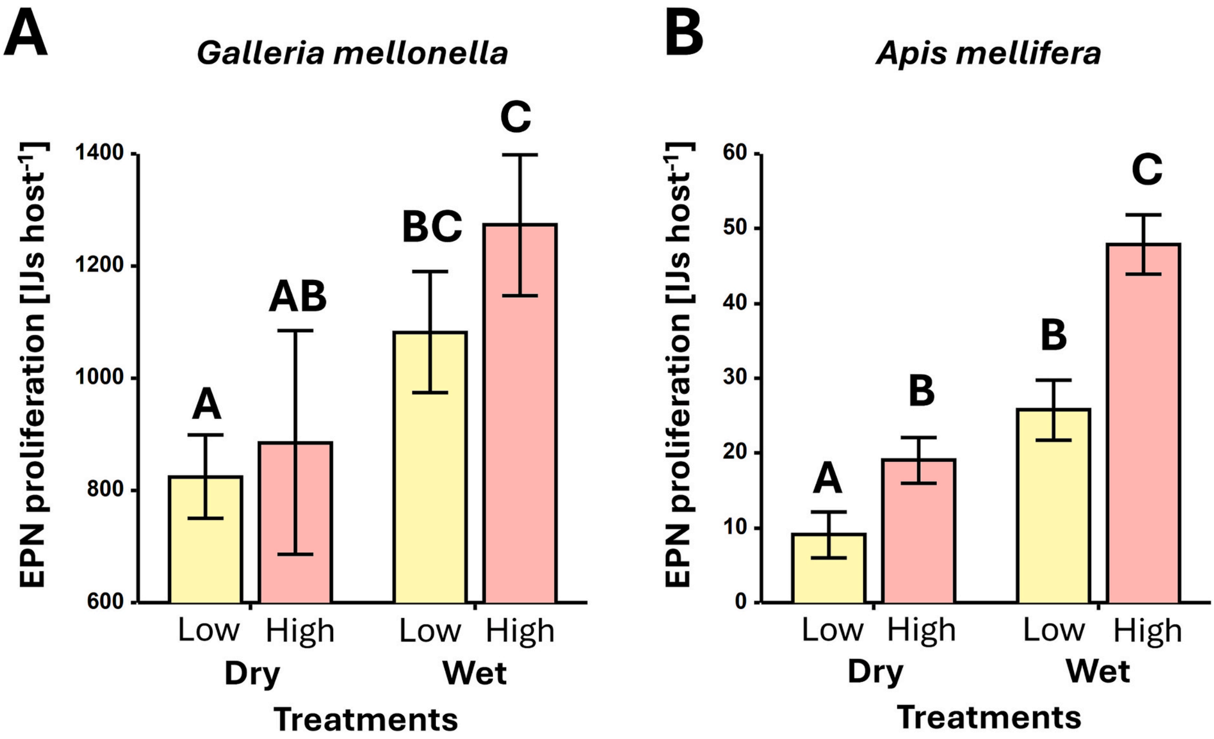

Fig. 3. Entomopathogenic nematode (EPN) proliferation visualized using error bar charts. The EPN proliferation was measured as infective juveniles (IJs) per host [IJs host^{-1}] in (**A**) greater wax moth larvae (*Galleria mellonella*) and (**B**) worker honey bees (*Apis mellifera*) exposed to varying doses (i.e., low and high) under different exposure scenarios (i.e., wet or dry). Both the dose as well as the exposure scenario significantly affect the infestation rates across both species (all *P*'s $<$ 0.02). The bar charts show both means (boxes) and standard errors (horizontal black bars and points) with the yellow and red bars representing low and high IJ exposure under dry and wet exposure scenarios, respectively. Significant statistical differences amongst treatments ($P < 0.05$) were identified using Bonferroni-corrected post-hoc pairwise comparisons and are denoted by lowercase letters (i.e., a, b, c).

the wet scenario increased infestation rates (Fig. 2). Likewise, an increasing dose of IJ exposure significantly affected the infestation rates across both species ($z = 5.40$; $P < 0.001$; Fig. 3). However, species-specific differences in infestation rates were observed. For *G. mellonella,* only the EPN wet and high treatment group significantly differed from the remaining treatment groups (all *z*'s $>$ 4.35; all *P*'s $<$ 0.001), revealing the highest infestation rate (100%) compared to an average infestation rate of 87% across the remaining treatment groups (Fig. 2). In contrast, for *A. mellifera*, the EPN wet and high treatment group (94%) revealed the highest infestation rate which significantly differed from the remaining treatment groups ($z = 6.11$; $P < 0.001$; Fig. 2). Further, the *A. mellifera* infestation rates for the EPN dry and high (77%) and EPN wet and low (66%) treatment groups did not significantly differ from one another ($z = -1.20$; $P = 1.0$; Fig. 3). Lastly, the *A. mellifera* EPN dry and low (26%) treatment group revealed the lowest infestation and differed significantly from all other treatments (all *z*'s $>$ 4.35; all *P*'s $<$ 0.001; Fig. 2).

3.3. IJ proliferation

Irrespective of the species, both application scenario as well as dose had a significant positive effect on the IJ counts (all *z*'s $>$ 2.48; all *P*'s $<$ 0.013). Furthermore, independent of the application scenario and dose, IJ counts were significantly higher in *G. mellonella* compared to *A. mellifera* ($z = 21.09$; all $P < 0.001$). For *G. mellonella*, the wet and high treatment group (1272 ± 114) revealed the highest IJ counts, yet no significant difference was observed when compared to the low and wet (1082 ± 100) treatment group (mean [n] ± S.E.; $z = 032$; $P = 1.0$; Fig. 3A). Likewise, the low and wet treatment did not significantly differ from the high and dry treatment (885 ± 161) ($z = 1.65$; $P > 0.59$), yet it did significantly differ from the low and dry (824 ± 101) (mean [n] ± S.E.; $z = 2.93$; $P < 0.02$; Fig. 3A). The *G. mellonella* dry and low did not significantly differ from the dry and high treatment group ($z = 0.32$; all $P = 1.0$; Fig. 3A). For *A. mellifera*, the dry and low treatment group significantly differed from all treatment groups ($z = 5.27$; $P < 0.001$; Fig. 3B), revealing the lowest IJ counts (9 ± 4; mean IJs per bee [n] ± S.E.). The dry and high (19 ± 3) and wet and low (25 ± 3) treatment groups did not significantly differ from one another (mean IJs per bee [n] ± S.E.; $z = 0.40$; $P = 1.0$; Fig. 3B). The *A. mellifera* wet and high treatment group revealed significantly different from all treatment groups ($z < 5.27$; $P < 0.001$; Fig. 3B), showing the highest IJ counts (47 ± 3; mean [n] ± S.E.).

4. Discussion

The data provide compelling evidence that foliar exposure to the EPN *Steinernema carpocapsae* significantly reduces survival of adult honey bees (*Apis mellifera*) under laboratory conditions. Under wet (direct overspray) and high-dose application conditions, mortality increased by nearly 70% relative to controls. These findings are consistent with previous reports of lethal effects of EPNs on adult bees following contact exposure (Shamseldean et al., 2004; Dutka et al., 2015) and demonstrate that commercially used EPN formulations can adversely affect non-target organisms, including pollinators (Bathon, 1996; Shapiro-Ilan et al., 2006; Erler et al., 2022). Collectively, these results suggest that current regulatory assessments for EPN products may not adequately capture potential risks to pollinators.

Our methodology, adapted from standardized chemical residue testing protocols (USEPA, 1996), revealed structural limitations when applied to living biological control agents such as EPNs. Control mortality in honey bees (~45%) and wax moth larvae (~22%) exceeded the recommended 20% validity threshold, indicating that protocols developed for synthetic chemicals are not readily transferable to organisms with different exposure routes and environmental requirements

(Candolfi et al., 2000; Erler et al., 2022). In wax moth larvae, elevated mortality likely reflects their dependence on high humidity and food availability, which are not adequately accommodated in standardized residue assays (Lacey and Georgis, 2012). In honey bees, mortality appeared linked to the early life stage tested and cage design rather than treatment effects per se. Newly emerged workers possess reduced cuticular lipids and weaker waterproofing (Lockey, 1988; Kather et al., 2011), increasing susceptibility to humidity-induced stress in enclosed exposure arenas. Additionally, some individuals may have failed to locate feeders mounted on cage lids and starved, despite ad libitum food availability. Collectively, these findings reflect systemic constraints of applying residue-testing frameworks to EPNs rather than incidental experimental shortcomings. Importantly, control survival exceeded 95% during the first 48 h - a critical period for EPN infection establishment (Shapiro-Ilan and Dolinski, 2015) – supporting the robustness of treatment effect interpretation.

To our knowledge, this study provides the first experimental evidence that foliar exposure to an EPN can significantly reduce adult honey bee survival. At treatment rates comparable to commercial application rates ($0.25–0.5 \times 10^6$ IJs m^{-2}), bee mortality reached 60–70% under wet-application conditions. In contrast, no significant effects were observed under dry-application conditions, highlighting that importance of surface moisture for EPN infection success. This pattern aligns with evidence that EPN viability and infectivity increase with humidity (Kaya et al., 1982; Nguyen and Smart Jr, 1991). For example, *S. carpocapsae* IJ survival remained above 60% at RH >70% and was still detectable after 16 h at ~40% RH (Ramakrishnan et al., 2022). In our study, IJ survival did not exceed 10 min once leaves were fully dry, yet this brief window was sufficient to infect exposed adult honey bees, while wet-application conditions likely prolonged IJ viability. Similar susceptibility has also been reported in *B. terrestris,* where exposure to EPN-contaminated soil resulted in approximately 80% mortality (Dutka et al., 2015).

Beyond infection, *S. carpocapsae* IJs successfully infest and proliferate in adult *A. mellifera* workers, albeit at significantly lower rates than in *G. mellonella*. Infestation rates in wax moth larvae remained consistently high (87–100%), whereas comparable values in honey bees occurred only under the high-dose, wet-application scenario (94%). These differences likely reflect fundamental host-traits. *Galleria mellonella* larvae are highly permissive hosts for IJs due to their thin cuticle, reduced immune defences, and accessible entry points, resulting in high IJ recovery (Poinar, 1993; Kaya and Gaugler, 1993). In contrast, foraging honey bees require IJs to adhere to the tarsi (Fig. 4) and locate spiracles while avoiding removal through grooming behaviour. Once inside the host, IJ proliferation differed markedly between species, with average recovery reached 1272 IJs per wax moth larva compared with 47 IJs per honey bee under high-dose, wet conditions. This disparity likely reflects differences in host size, nutrient availability, immune defences, and initial IJ penetration (Baur et al., 1995; Shapiro-Ilan and Lewis, 2024). Proliferation rates in honey bees were substantially lower than those reported for bumble bees exposed via soil (Dutka et al., 2015), likely due to differences in exposure conditions, nematode species, and post-application drying. These findings emphasize that host body traits, nematode biology, and exposure conditions jointly shape infection and proliferation outcomes (Blanco-Pérez et al., 2017, 2019; Fowler et al., 2020). Future comparative studies using standardized EPN species and harmonized exposure routes are needed to disentangle these effects. In addition, the comparatively low IJ production per host, particularly in *G. mellonella* (e.g., Metwally et al., 2012; Campos-Herrera et al., 2015), is likely linked to the experimental design, where reduced IJ survival—especially under dry conditions—constrained infection success and subsequent nematode reproduction.

EPNs are frequently classified as minimal-risk products due to their natural origin, soil-associated ecology, and perceived host specificity (Kaya and Gaugler, 1993; Blanco-Pérez et al., 2024; Campos-Herrera et al., 2025), and therefore commonly exempted from pollinator safety testing. Our results challenge this assumption by demonstrating that commercially used *S. carpocapsae* formulations can cause substantial honey bee mortality under realistic foliar exposure conditions. While regulatory frameworks for synthetic pesticides are well established (USEPA, 2012; EFSA, 2023), standardized pollinator risk assessments for EPNs and other biological control agents remain limited. Despite their continued value in integrated pest management, particularly against soil-dwelling pests (Kaya and Gaugler, 1993; Blanco-Pérez et al., 2024), our findings indicate that foliar applications involve ecological trade-offs that warrant careful consideration. Reduced infestation and mortality under dry-application conditions likely reflected rapid IJ death after leaf drying rather than intrinsically lower susceptibility. In field settings, canopy structure, shaded foliage, and elevated RH during nighttime or early morning hours can prolong IJ survival (Ramakrishnan et al., 2022), potentially increasing exposure risk for non-target organisms.

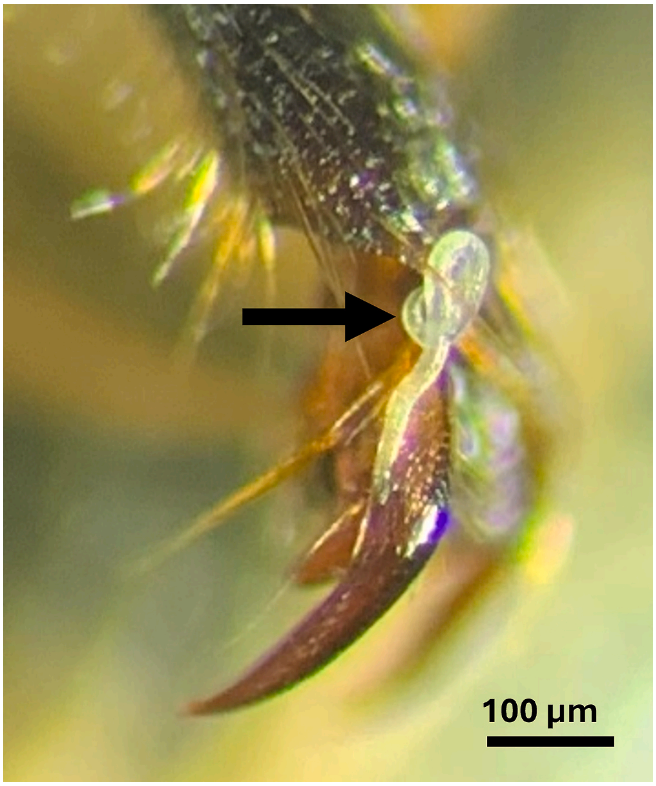

Fig. 4. Entomopathogenic nematode (EPN) detection post application of a worker honey bee (*Apis mellifera*). Light microscopy (10× magnification) showing an infective juvenile (IJ) *Steinernema carpocapsae* attached to the tarsus of a worker honey bee following an EPN exposure via foliar application. The black arrow indicates the IJ.

5. Conclusion

Our results underscore the need for further research on the potential adverse effects of EPNs and other biological pest control agents on non-target organisms (Erler et al., 2022). Although infestations in natural settings are likely to occur at much lower levels, these findings demonstrate that EPNs should not be considered inherently low-risk without dedicated non-target evaluation (Hokkanen et al., 2003; Ramakuwela et al., 2025). This is particularly relevant for soil-dwelling bee species - which represent the majority of bee diversity (Engel et al., 2021) - as both adults and brood may encounter IJs in treated soils or during foraging on flowering crops. However, while laboratory and semi-field studies demonstrate that bees can be infected under defined exposure scenarios (Shamseldean et al., 2004; Dutka et al., 2015), field-based data on natural infection prevalence and exposure frequency remain scarce (Erler et al., 2022; Katlav et al., 2025), limiting direct extrapolation to real-world risk.

While EPNs are increasingly recognized as promising, environmentally friendly alternatives to chemical pesticides, their broader adoption remains constrained by key knowledge gaps related to application, risk assessment and regulation (Campos-Herrera et al., 2025). Our laboratory results highlight that, despite their biological origin, EPNs should not be assumed to be inherently safe, and potential non-target effects must be considered and if necessary, carefully evaluated. Addressing these challenges will require stronger collaboration between academia, industry and regulatory bodies to generate robust data, improve testing frameworks, and ensure that EPN based products are both effective and environmentally safe (Londoño and Frettinger, 2024). To support their safe deployment, we advocate the development of standardized regulatory study protocols for biological control agents that include pollinator-focused testing and realistic exposure scenarios for the risk-assessment, consistent with recommendations for microbial pesticides (Borges et al., 2021).

Where foliar applications are used, restricted applications to night-time hours (i.e., after dusk) - when pollinator activity is typically reduced – should be considered as a precautionary risk-mitigation measure to limit non-target exposure under wet conditions. This approach aligns with existing plant protection product mitigation practices (USEPA, 2012; The Commission of the European Communities, 2003) and may simultaneously enhance EPN efficacy under humid, low-UV conditions while reducing pollinator risk. Such measures are essential to balance effective pest management with pollinator conservation and to preserve the ecological benefits of biological control (Campos-Herrera et al., 2025).

CRediT authorship contribution statement

Lars Straub: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Angélique Rüfenacht:** Writing – review & editing, Investigation, Data curation. **Daniela Grossar:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Conceptualization. **Lukas Jeker:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2026.181861.

Data availability

Data available via the Dryad Digital Repository: http://datadryad.org/share/vXEhuGt6-Yq9dF2MQtxHCVL-rEnqoXFZcg0NmHwwksU.

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