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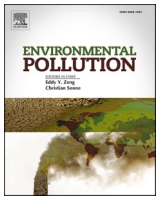

Chronic oral toxicity protocol for adult solitary bees (*Osmia bicornis* L.): Reduced survival under long-term exposure to a "bee-safe" insecticide☆

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A R T I C L E I N F O

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A B S T R A C T

Pollinators are essential for crop productivity. Yet, in agricultural areas, they may be threatened by pesticide exposure. Current pesticide risk assessments predominantly focus on honey bees, with a lack of standardized protocols for solitary bees. This study addresses this gap by developing a long-term oral exposure protocol tailored for *O. bicornis*. We conducted initial trials to determine optimal container sizes and feeding methods, ensuring high survival rates and accurate syrup consumption measurements. A validation test involving five laboratories was then conducted with the insecticide Flupyradifurone (FPF). Control mortality thresholds were set at ≤ 15% at 10 days. Three laboratories achieved ≤10%, demonstrating the protocol's effectiveness in maintaining healthy test populations. The seasonal timing of experiments influenced control mortality, underscoring the importance of aligning tests with the natural flight period of the population used. Our findings revealed dose-dependent effects of FPF on syrup consumption, showing stimulatory effects at lower concentrations and inhibitory effects at higher ones. The 10-day median lethal daily dose (LDD50) of FPF for *O. bicornis* (531.92 ng/bee/day) was 3.4-fold lower than that reported for *Apis mellifera* (1830 ng/bee/day), indicating *Osmia*'s higher susceptibility. Unlike other insecticides, FPF did not exhibit time-reinforced toxicity. This study introduces a robust protocol for chronic pesticide exposure in solitary bees, addressing a critical gap in current risk assessment. Based on its low risk to honey bees and bumblebees, FPF is approved for application during flowering. However, our results suggest that it may threaten *Osmia* populations under realistic field conditions. Our findings underscore the need for comparative toxicity studies to ensure comprehensive protection of all pollinators and the importance of accounting for long term exposure scenarios in risk assessment. By enhancing our understanding of chronic pesticide effects in solitary bees, our study should contribute to the development of more effective conservation strategies and sustainable agricultural practices.

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1. Introduction

Declines in bee diversity and abundance over the last decades have been widely documented at both local and regional scales (Bartomeus et al., 2013; Biesmeijer et al., 2006; Ollerton et al., 2014; Zattara and Aizen, 2021). Although these declines have multiple causes, the use of pesticides is considered one of the major contributing factors (Goulson et al., 2015; Sánchez-Bayo and Wyckhuys, 2019). Prior to their approval for commercial use, pesticides undergo a risk assessment process to ensure their use will not pose a threat to non-target organisms, including bees (Sgolastra et al., 2020). Bee risk assessment follows a tiered approach, progressing from simpler assessments in the laboratory (tier 1), to more realistic assessments in semi-field and field (higher tier) conditions. When laboratory tests, conducted under worst-case exposure conditions, indicate a high potential risk, higher tier experiments are conducted (Sgolastra et al., 2020).

Pesticide regulation is an essential component of pollinator protection programs. However, pesticide risk assessment schemes have some limitations and, therefore, are permanently being revised to increase the level of protection (EFSA, 2013; EFSA et al., 2023; Sgolastra et al., 2020). One of these limitations is an insufficient coverage of chronic exposure. Although chronic toxicity tests are available (see below) and their incorporation has been emphasized in the guidance documents of the European Food Safety Authority (EFSA, 2013; EFSA et al., 2023), these have not been officially endorsed at the EU level. As a result, authorisation decisions still rely on acute toxicity data, even though in field conditions bees are usually exposed to pesticides for extended periods (Azpiazu et al., 2023b; Botías et al., 2015; Tosi et al., 2018). Therefore, it is crucial to include tests of chronic exposure to obtain a comprehensive understanding of the long-term consequences of continued exposure. In addition to addressing dose-dependent effects, chronic exposure tests should also address accumulative toxicity effects due to long-term exposure. Accumulative toxicity occurs when prolonged exposure to low doses produces a greater effect than a short-term exposure to an equivalent higher dose. In other words, when the toxic effects of a chemical are reinforced by exposure time. Accumulative toxicity can be measured following time-reinforced toxicity (TRT) approaches (EFSA et al., 2023; Tosi et al., 2021). TRT effects are caused by the bioaccumulation of the pesticide in the body of the organism (Rondeau et al., 2015; Simon-Delso et al., 2018). The EFSA has recently proposed the inclusion of TRT measures in tier 1 of bee risk assessments (EFSA et al., 2023)

An additional limitation of current bee risk assessment schemes is that they rely solely on the honey bee, *Apis mellifera*, assuming this species is a good surrogate for other bees (Franklin and Raine, 2019). However, bees (Hymenoptera, Anthophila) comprise more than 20,000 species worldwide (Michener, 2007), encompassing a wide range of life history traits. Unlike honey bees, which are social, solitary bees (and their cleptoparasites) account for almost 90% of bee species (Danforth et al., 2019). As a consequence of this and other differences in life history traits, certain pathways of pesticide exposure that are particularly relevant to solitary bees but not honey bees are not adequately addressed in existing risk assessment schemes (Sgolastra et al., 2019). Moreover, different bee species exhibit varying degrees of sensitivity to different classes of pesticides (Arena and Sgolastra, 2014; Azpiazu et al., 2021; EFSA et al., 2023; Linguadoca et al., 2022; Pamminger, 2021; Sgolastra et al., 2017; Uhl et al., 2016). Considering these factors and recognizing the difficulty to extrapolate pesticides effects from honey bee endpoints to other bees, the European Food Safety Authority emphasized the need to include solitary bees, such as *Osmia cornuta* and *Osmia bicornis*, in risk assessment schemes (EFSA, 2013).

A standardized protocol to assess the effects of 10-day exposure to pesticides on honey bees is available since 2017 (OECD, 2017a), and a protocol to evaluate the impact of long-term (more than 10 days) chronic exposure has been recently ring-tested (Tosi et al., 2021). Standardized risk assessment protocols are yet to be established for *Osmia* spp., but some protocols are being developed and/or ring-tested (Cabrera et al., 2024; Roessink et al., 2017). In addition, various studies have used different methodologies to study pesticide effects on *Osmia* adults, including acute topical exposure (Bednarska et al., 2022; Biddinger et al., 2013; Hayward et al., 2019; Scott-Dupree et al., 2009), acute oral exposure (Albacete et al., 2024, 2023; Azpiazu et al., 2023a, 2021; Ladurner et al., 2005a, 2003; Linguadoca et al., 2022; Phan et al., 2020; Sgolastra et al., 2018, 2017) and chronic oral exposure (Azpiazu et al., 2023a, 2022; 2019; Boff et al., 2021; Heard et al., 2017; Mokkapati et al., 2022; Robinson et al., 2017; Spurgeon et al., 2016; Strobl et al., 2021). In this study, we introduce a chronic oral toxicity protocol for *Osmia,* based on the chronic exposure methodology currently used for honey bees adjusted to the biology and behaviour of *Osmia*. Then, to validate the protocol, we provide independent results from five laboratories testing the chronic oral toxicity of the insecticide flupyradifurone (FPF) on *O. bicornis*. We calculate the median lethal daily dose (LDD50) at 10 days and assess the effects of sublethal chronic oral exposure on syrup consumption. We also assess potential accumulative toxicity effects resulting from long-term exposure (time-reinforced toxicity).

FPF is a butenolide insecticide developed by Bayer CropScience and marketed under the name Sivanto® (Nauen et al., 2015), approved by the European Union in 2015. Similarly to neonicotinoids recently banned in the EU (Brown et al., 2016; Sgolastra et al., 2020), FPF is a systemic insecticide that acts as an agonist of nicotinic acetylcholine receptors (nAChR) (Nauen et al., 2015). Based on its low risk to social bees (honey bees and bumblebees) the use of FPF is allowed during bloom (Nauen et al., 2015; USEPA, 2014), even though subsequent studies unveiled various sublethal effects (Gray et al., 2024; Hesselbach et al., 2020; Richardson et al., 2024; Siviter and Muth, 2022; Tan et al., 2015; Tosi et al., 2021). The scarce evidence available suggests that FPF may pose a greater risk to solitary bees. The alfalfa leafcutting bee, *Megachile rotundata*, was found to be 30 to 170 times more sensitive to FPF contact exposure than honey bees and bumblebees (Hayward et al., 2019), and exposure to sprays at field application rates resulted in decreased survival in the blue orchard bee, *Osmia lignaria* (Siviter et al., 2024). When combined with a fungicide, FPF was found to decrease the number of emerging offspring in ground-nesting squash bees, *Xenoglossa pruinosa* (Rondeau and Raine, 2024).

2. Materials and methods

2.1. Preliminary study: test cage assessment

In 2020, five laboratories conducted a preliminary trial to establish an appropriate cage type in which to perform the exposure phase with *O. bicornis*. We tested Nicot cages (also known as roller queen cages; Nicotplast SAS, France), which are commonly used in bumble bee acute toxicity tests, and plastic containers (see Supplementary Information). Plastic containers were larger (150 cc) than Nicot cages (25 cc). Adding a petal to the syrup dispenser (syringe) has been shown to facilitate prompt location of the syrup source (Azpiazu et al., 2023a). For this reason, we tested large cages with and without a flower petal of *Euryops* (Asteraceae) attached to the tip of the feeding syringe (Fig. S1; Azpiazu et al., 2023a). Large cages with the petal performed better (feeding success: 89.2 %; bee longevity: 24.7 $\pm$ 1.1 days) than large cages without the petal (feeding success: 65.3 %; bee longevity: 22.6 $\pm$ 1.3 days), or Nicot cages (feeding success: 53.2 %; bee longevity: 21.2 $\pm$ 1.2 days) (Table S1). The conclusions from this preliminary test were that Nicot cages were unsuitable for long-term exposure experiments with *Osmia* and that the addition of the petal significantly facilitates the prompt location of the feeder (see Supplementary Information for statistics and additional data).

2.2. Chronic exposure test

In 2022, another group of five laboratories conducted the actual chronic exposure toxicity test using a common methodology as described below (see Supplementary Information for the detailed protocol).

2.3. Osmia bees and test conditions

The tests were conducted with newly emerged *O. bicornis* females. Different laboratories used populations reared in natural areas in their respective countries. In autumn, once all individuals had reached the adult stage within their cocoons, the cocoons were transferred to 3–4 °C chambers for wintering. In spring, large cocoons (presumed to contain females) were incubated at 22-23 °C until emergence. The timing of incubation and emergence differed among laboratories (Table 1). Bees were maintained under laboratory conditions throughout the test: temperature (mean: 25–28 °C; Table 1), ambient humidity (40–60%) and indirect natural light. Temperature and relative humidity (RH) were recorded daily.

2.3.1. Test procedure and experimental cages

Cocoons were checked daily for emergence throughout the incubation period. Upon emergence, bees were transferred to a flight cage (>50 × 50 × 50 cm) for meconium deposition and starvation. Twenty-four hours later, bees were individually transferred to plastic cages (volume = 500 cc; height: 70 mm; Ø: 110 mm) with a transparent pin-perforated lid to allow air exchange. Each cage had a 1-ml calibrated syringe with the tip cut off inserted laterally at approximately 35 mm from the base. Adhesive tape attached to the outer walls of the container was used to secure the syringe in a slightly tilted position, thus facilitating syrup flow. A petal of *Euryops* sp. was attached to the end of the syringe to help the bee locate the feeder. On subsequent days, once the bee had learnt the position of the feeder, the petal was removed. Syringes were covered with a sheath of black cardboard to avoid pesticide degradation. Twenty-four hours after the introduction of the bees in the cages we assessed syrup consumption by checking the level of test solution in the syringe. Only bees that fed at least 10 μl within the first 24 h of exposure were considered successful feeders and were retained for the duration of the chronic test. Syrup consumption and survival were assessed daily until the last bee died. To account for potential evaporation of the feeding solution, three additional cages without bees were also monitored. Initial samples sizes per laboratory were at least 30 bees per treatment divided into three groups (10 bees per group) corresponding to three incubation times one week apart from each other.

2.3.2. Flupyradifurone and dimethoate concentrations and feeding solution

The syringe feeders were filled with a sucrose solution (33% w/w) with or without insecticide and bees were allowed to feed ad libitum throughout the test. The insecticide was dissolved into the sucrose solution at the desired concentrations. We had seven treatments: a negative control (sucrose water solution, 33% w/w), a positive control (1 mg/L of dimethoate) (OECD, 2017a), and five different concentrations of FPF (0.77, 1.92, 48.00, 12.00, 30.00 mg/L; geometric series factor of 2.5).

New feeding solution was prepared every seven days and stored in darkness at 6 ± 2 °C to prevent photolysis and degradation. The feeding solution in the syringes was renewed every 3–4 days to avoid proliferation of microorganisms.

The stock solutions of both FPF (CAS #: 951659-40-8, Purity: 99.9%, PESTANAL analytical standard, Sigma-Aldrich Laborchemikalien GmbH) and dimethoate (CAS #: 60-51-5, EC #: 015-051-00-4, Purity: 99.5%, PESTANAL analytical standard, Sigma-Aldrich Laborchemikalien GmbH) were prepared with distilled water at the beginning of the test and stored in a refrigerator (6 ± 2 °C). We avoided the use of acetone because both pesticides are soluble in water at the stock concentrations (FPF: 3200 mg/L, dimethoate: 25900 mg/L).

To evaluate the stability of FPF, we measured its concentration using ultra-high-pressure liquid chromatography (UHPLC, Agilent 1290 series with 6470 Triple Quad, Agilent Technologies). This analysis was performed on both the initial stock solution and the feeding solution. Two different concentrations were measured for each matrix (stock solution: 15 and 600 mg/L; feeding solution: 0.8 and 30.5 mg/kg). These solutions were stored under refrigerated conditions (6 ± 2 °C). The stability of the feeding solution within the syringe feeders was also examined. For the stock solution, measurements were conducted on day 0 and after four weeks. The feeding solution, stored in the refrigerator, underwent measurements at two distinct time points: upon preparation (day 0) and after three days (day 3), which coincided with the refilling of the syringes. Furthermore, the feeding solution extracted from the syringes, simulating conditions within cages, was assessed with and without black cardboard on day 3 (initial syrup refill) and day 7 (renewal of feeding solution). Three replicates were analysed per day and concentration. According to SANTE/2020/12830 guidelines, Rev. 2 (EU, 2023), product concentration is stable when the variation of concentration between the fresh samples and the aged samples is <20%. Details of analytical techniques and results are provided in Supplementary Information. FPF concentrations measured at two different times in the stock and feeding solutions, both stored in the fridge and in the syringe feeders, confirm that the active ingredient FPF can be considered stable under the test conditions (Table S2). The variation in concentration was lower than 20% in all cases. According to OECD guidelines, a concentration is stable when it remains within 80–120% of nominal or mean measured values over the entire exposure period.

Table 1
Experimental conditions and control mortality results in the five laboratories participating in the validation test for the assessment of FPF toxicity under chronic oral exposure in *O. bicornis* females.

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
Test start date	09/05/2022	15/05/2022	15/05/2022	25/05/2022	04/06/2022
Mean temperature ± SE (°C)	25.9 ± 0.25	–	28.02 ± 0.23	24.24 ± 0.21	27.11 ± 0.18
% Control mortality at 10 days	5.8%	10.7%	10.7%	16.70%	31.0%
Validity criteria[a]	Fulfilled	Fulfilled	Fulfilled	Failed	Failed
Control LT50 (days)[b]	24	34	38	24	19

[a] Control mortality at 10 days <15% (OECD, 2017a,b).
[b] Lethal time 50 (time required for 50% of the study population to die).

2.4. Statistical analysis

2.4.1. Survival

We used Kaplan–Meier (K-M) survival curves to illustrate the combined effects of insecticide concentration treatments on survival. Then, we ran a log-rank omnibus test to explore overall differences among concentrations (*survdiff* function of the survival R package with $\rho = 0$; Therneau et al., 2020). Pairwise comparisons between survival curves were done with Holm multi-comparison corrections and $\rho = 0$ (pairwise_survdiff function of the *survminer* package; Kassambara et al., 2020).

2.4.2. Calculation of median lethal daily dose at 10 days

To estimate the median Lethal Daily Dose (LDD50) at 10 days, we first calculated the daily dose [μg/bee/day] of the concentrations tested [mg/L] using the average of the daily bee consumption [μl solution/bee/day] during the first 10 days of the experiment. Then, dose-response models were fitted to mortality data at the different exposure doses using the *drc* package (Ritz et al., 2015). The *mselect* function of *drc* was used to determine which models used for binomial response data (i.e.

3-parameter models) were most appropriate based on the Akaike information criterion (AIC). LDD50 and lethal daily dose for 10% of the population (LDD10) doses were then estimated with the *ED* function of *drc* package. Following the validity criteria of OECD protocol for honey bees, we built dose-response curves and calculated the overall LDD50 and LDD10 considering only the data of the (three) laboratories in which control mortality was <15% (at 10 days). We calculate a critical reference point, considered the lowest dose that produces an adverse response compared to the negative control, as the 95% lower confidence bound of the LDD10. We use this approach instead of the traditional no-observed-effect-level (NOEL) method because NOEL is highly dependent on dose selection and sample size (Davis et al., 2011).

2.4.3. Syrup consumption

We used a linear mixed model (LMM) to analyse log-transformed daily syrup consumption during three periods (days 1–10, 11–20 and 21–30). We included FPF concentration (0.768, 1.92, 48.00, 12.00, 30.00 mg/L) as fixed factor, and laboratory (Lab 1, Lab 2 and Lab 3) as random factor. We tested the significance of the main effects with the likelihood ratio test ($p < 0.05$).

2.4.4. Time reinforced toxicity

We applied the standard, most recent methodology (EFSA et al., 2023) to investigate the potential of FPF to exert time reinforced toxicity (TRT). The TRT behaviour was estimated from the first 10 days of the experiment using the dose data at each concentration tested as explain above. For each laboratory, data were fitted to the reduced version of the General Unified Threshold models of Survival (GUTS-RED) model, using both the Stochastic Death (SD) and the Individual Tolerance (IT) approaches. From the IT and SD fitted models, LDD50 values were estimated for every day until day 27. Later, the LDD50 at day 10 and day 27 were compared. In agreement with the recommendations of EFSA et al. (2023), TRT was concluded if the median LDD50 at day 27 was lower by at least a factor of 2.7 with respect to the LDD50 at 10 days. The target of 27 days is specific for honey bee workers, corresponding to the median field adult lifespan (EFSA et al., 2020). The average daily mortality of *O. bicornis* ranges between 0.028 and 0.09, corresponding to an adult lifespan of 11–35 days (EFSA et al., 2020), however we maintained the standard value of 27 days as a worst-case scenario. The robustness of the adopted methodology was checked by extrapolating survival trends calibrated over 10 days to the entire study period. We assumed that the longevity of a cohort of synchronized-emerging bees shows a unimodal pattern. To check this assumption, longevity distributions in the controls were fitted to standard unimodal distributions (namely normal, Weibull, gamma, logistic, and log-normal). The best fit based on the Akaike Information Criterion (AIC) was selected for the following step of the analysis (Table S6).

2.4.5. Potential risk of O. bicornis under field conditions

We calculated a risk index (RI) to compare the hazard posed by FPF levels found in different crops (melon, cotton, blueberry, and apple; USEPA, 2014) to *O. bicornis*. Following Sgolastra et al. (2024), the RI combines estimated exposure concentrations, daily amounts of nectar ingested and the lowest dose that produces a lethal effect (LDLE). RI values close to 1 indicate potential lethal risk for bees.

For each crop and bee species the RI was calculated as:

$$RI = \frac{Rn^{*}NC}{LDLE}$$

where: *Rn* is the FPF maximum level of residue found in nectar (USEPA, 2014, Table 3) expressed in mg/Kg; *NC* is the daily nectar consumption in mg/day; and LDLE is the lower confidence bound of the LDD10 calculated in this study (256.8 ng/bee/day). Nectar consumption (NC) was calculated based on EFSA's (2012) estimate of daily sugar intake in *Osmia* (77 mg/day), adjusted by the nectar % sugar concentration of each crop.

3. Results

3.1. Survival curves and median lethal daily dose 50 (LDD50)

As expected, the mortality of females exposed to dimethoate (positive control) exceeded 50% within 10 days in all laboratories. The cumulative survival curves of *O. bicornis* females chronically exposed to FPF showed significant differences across concentrations in all laboratories (Log Rank test; Fig. 1; Table S3). The validity criterion of mortality of the control group (<15%) was met in three of the five laboratories (Table 1).

Table 3
Risk index (RI) for *O. biconis* based on maximum FPF residues measured in the nectar of several crops treated during bloom (USEPA, 2014).

Crop	FPF concentration (mg/kg)	Sugar concentration (%)	*O. bicornis* RI
Melon	0.36	20[a]	0.49
Cotton	0.39	53[b]	0.22
Blueberry	0.64	42[c]	0.46
Apple orchard 1	1.2	42[d]	0.86
Apple orchard 2	1.5	42[d]	1.07

[a] Dag and Eisikowitch (1999).
[b] Gottsberger et al. (1984).
[c] Bożek (2021).
[d] Butler (1945).

Table 2
Median lethal daily dose (LDD50), lethal daily dose for 10% of the population (LDD10) and their 95% confidence intervals at 10 days in *O. bicornis* females subjected to chronic oral exposure to FPF in five laboratories.

	Lab 1	Lab 2	Lab 3	Lab4	Lab5	Overall[a]
n	194	165	173	181	173	532
Body weight (mg) ± SE	102.61 ± 0.47	85.99 ± 0.54	93.26 ± 1.25	89.53 ± 4.41	86.97 ± 0.61	93.56 ± 0.62
Model	Weibull	Normal	Logistic	Normal	Normal	Logistic
AIC	24.58	21.77	22.24	21.69	25.47	28.6
t-value	16.61	41.68	49.88	22.21	1.56	17.59
p-value	<0.001	<0.001	<0.001	<0.001	0.119	<0.001
LDD50 (ng/bee/day)	504.27	442.131	586.94	302.38	–	531.92
95% CI	435.62–572.93	360.44–523.82	489.17–684.70	258.86–345.90	–	472.67–591.18
LDD50 (ng/mg/day)	4.91	5.14	6.29	3.38	–	5.69
95% CI	4.25–5.58	4.19–6.09	5.25–7.73	2.89–3.86	–	5.05–6.32
LDD10 (ng/bee/day)	339.51	260.29	383.74	220.60	–	316.01
95% CI	291.33–387.69	177.63–342.94	281.55–485.92	153.08–288.12	–	256.81–375.20
LDD10 (ng/mg/day)	3.31	3.03	4.11	2.46	–	3.38
95% CI	2.84–3.78	2.07–3.99	3.02–5.21	1.71–3.22	–	2.74–4.01

[a] Considering only data from labs that met the validity criterion (Labs 1, 2 and 3).

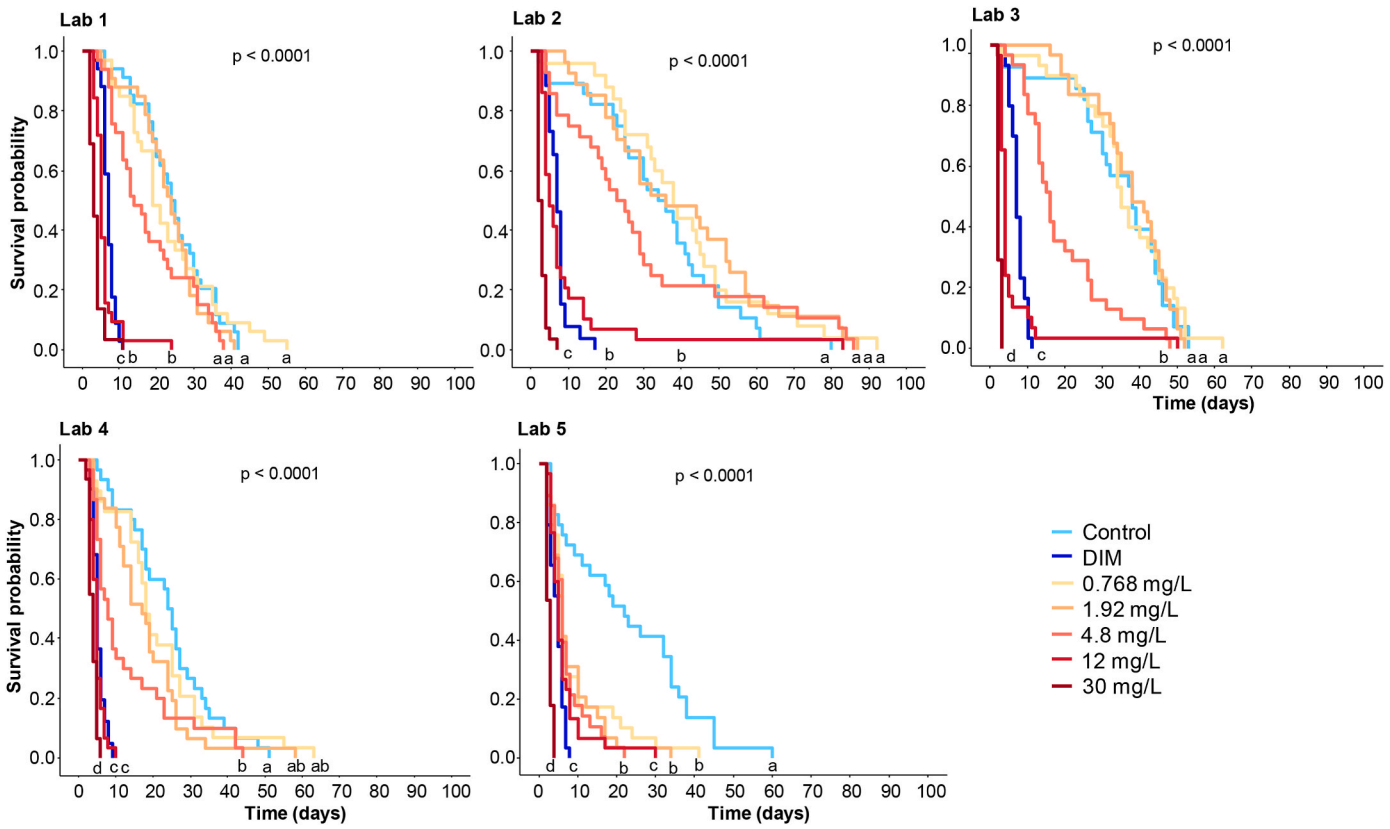

Fig. 1. Survival probability of *O. bicornis* females chronically exposed to different FPF concentrations and to the reference toxic standard dimethoate (DIM, at 1 mg/L) in five laboratories. Different letters denote significant differences (Holm pairwise comparisons).

The mean daily dose of FPF consumed during the first 10 days by bees of the different treatments is shown in Table S4. In four of the five laboratories, the dose-response relationships were significant (Table 2). The 10-day LDD50 values obtained in the laboratories that met the validity criterion (Labs 1, 2 and 3) were similar as indicated by the overlapping confidence intervals (Table 2). Similarly, the 10-day LDD10 values from Labs 1, 2, 3, and 4 also showed similar results due to the overlapping confidence intervals (Table 2). The overall estimated lowest doses that produce an adverse response compared to the negative control, as determined by the 95% lower confidence bound of the LDD10 was 256.81 ng/bee/day (Table 2).

3.2. Syrup consumption

Considering only the data from laboratories that met the validity criterion (Labs 1, 2 and 3), syrup consumption varied significantly across FPF concentrations during the first two time periods assessed (LMM; 1–10 days: $F = 15.24$, $df = 5$, $p < 0.001$; 11–20 days: $F = 14.93$, $df = 4$, $p < 0.001$), but not during the third period, when only control bees and bees exposed to the lower insecticide concentrations remained alive (21–30 days: $\chi 2 = 1.53$, $df = 3$, $p = 0.207$). Bees exposed to the highest FPF concentration showed decreased daily syrup consumption compared to the control (Fig. 2). On the other hand, bees exposed to the

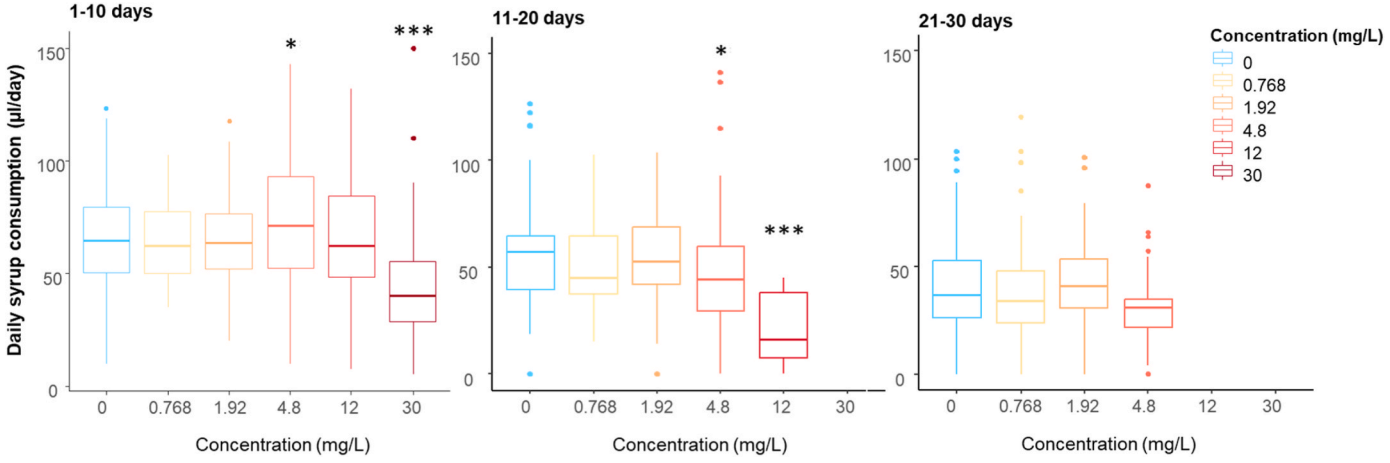

Fig. 2. Mean daily syrup consumption ± SE (µl/day/bee) during three 10-day periods in *O. bicornis* females chronically and orally exposed to five FPF concentrations. Asterisks indicate significant differences between the FPF concentration (ng/g) and the control (LMM; *p < 0.05 **p < 0.001, ***p < 0.001). Only data from laboratories meeting the validity criterion were used.

intermediate FPF concentration (4.8 mg/L) consumed more syrup than control bees during the first period (Fig. 2). Details of mean daily syrup consumption at the different FPF concentrations in each laboratory and time period are provided in Fig. S2.

3.3. Time reinforced toxicity

TKTD (GUTS) fitting: Both GUTS-SD and GUTS-IT provided very good to excellent fit with the data (Figs. S3–S7). Both models provided similar temporal patterns of survival over the 10 days and largely overlapping credible parameter intervals across labs (Figs. S3–S7). However, both models overestimated background mortality rate for Lab 5 (Fig. S8). Background mortality rate clearly differed between Lab 5 and the remaining laboratories. Both SD and IT models were thus re-fitted using a fixed background mortality rate estimated by fitting survival data of the control to a single first-order decay model (resulting decay constant: 0.045 day^{-1}). These fitted results, which remained similar, were used in the final analysis. The GUTS-SD and GUTS-IT results (Figs. S9–S10) validate the robustness of the experimental data (except for Lab 5) and the TKTD model results.

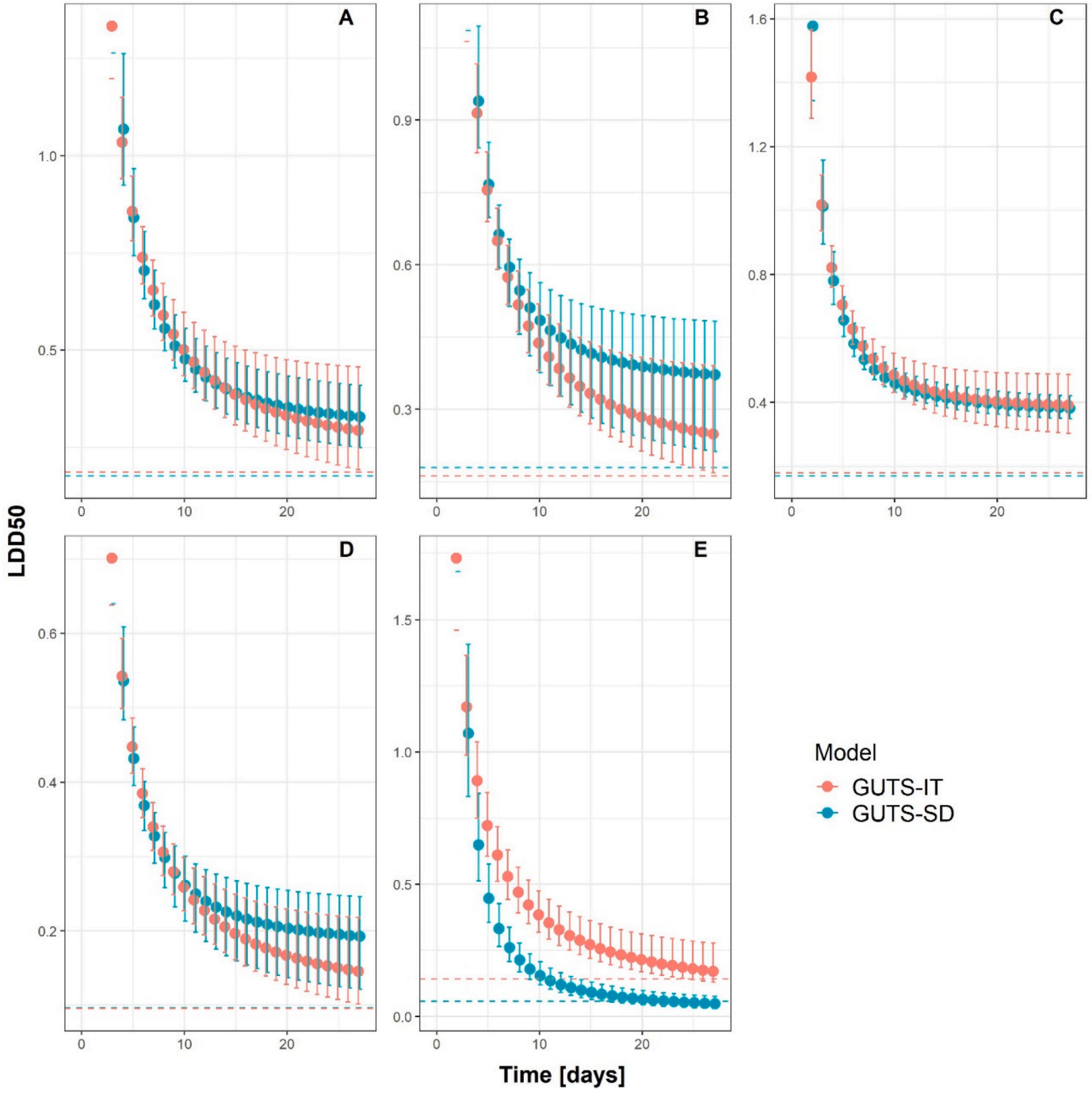

Fig. 3. LDD50 in mg/bee (and related 95% credible interval) trend over time for all laboratories. The results were predicted using GUTS-SD (red) and GUTS-IT (blue) models. The dashed horizontal lines represent the thresholds for TRT, corresponding to the LDD50 at 10 days divided by 2.7 (see Methods section for details). The labs are (A) Lab 1, (B) Lab 2, (C) Lab 3, (D) Lab 4, (E) Lab 5 (background mortality fitted separately and fixed). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

TRT assessment: The results demonstrate that FPF does not show TRT properties in *O. bicornis*. Compared to exposure level, time of exposure plays a smaller influence than expected from Haber's law (Miller et al., 2000). In fact, the median LDD50 at 27 days was higher than the LDD50 at 10 days divided by 2.7 (Fig. 3). This is consistent across models and laboratories, except Lab 5. This Lab showed credible limits of the LDD50 at 27 days below the threshold under both models. This is likely due to the large uncertainty in model parameters, propagating into large uncertainty of survival estimates and thus to unreliable LDD50 estimates for Lab 5. This dataset has however lower fit than all others (see TKTD fitting section), limiting the reliability of this outcome.

Extrapolation of survival trends: The extrapolation of the survival trends calibrated in the first 10 days to the entire study period further demonstrate the robustness of our methodology (Fig. S11). The models calibrated over the first 10 days of the test predict survival at the tested doses for the full duration of the experiment reasonably well (Figs. S12–S15). The only exception to this general trend is again Lab 5 (Figs. S16–S17). This is not unexpected considering the constraints of the results of this laboratory.

The analysis consistently indicates lack of TRT properties in *O. bicornis* chronically exposed to FPF. Predictions for the survival trends beyond 10 days also showed that the models were generally able to accurately describe the outcome of the tests (except for Lab 5). Of the five labs, only one had lower fit due to high control mortality and lack of a clear dose-response pattern, producing less reliable outcomes.

3.4. Potential risk of O. bicornis under field conditions

Risk levels varied across crops (Table 3). The RI was close to 1 on apples, indicating potential lethal risk (Table 3).

4. Discussion

In this study, we adapted existing honey bee chronic exposure protocols (Tosi et al., 2021) to the biology and behaviour of *Osmia*. We first conducted a preliminary trial to establish an appropriate cage size for individual exposure. Nicot cages proved unsuitable for long-exposure tests with *Osmia*. Frequent contact between the body of the bee and the tip of the syringe causes bees to become daubed in syrup, resulting in low survival and hindering the measurement of syrup consumption (Azpiazu et al., 2023a). The larger containers, on the other hand, provided longevities comparable to those of field bees (Bosch and Vicens, 2006) and allowed for accurate measurement of syrup consumption. Unlike honey bees and bumblebees (OECD, 2017a; 2017b, 1998), *Osmia* spp. are usually reluctant to feed on artificial feeders (Azpiazu et al., 2023a; Ladurner et al., 2005b, 2003). For this reason, we attached a petal to the tip of the syringe to facilitate prompt location of the feeder, thus reducing the number of unfed bees and variability among fed bees in the timing of exposure (Azpiazu et al., 2023a), which allowed us to work only with bees that began feeding within 24 h. All participating labs obtained feeding success rates higher than 80% (Table S5).

The quality of the test population is a fundamental parameter in any toxicity study. Standard chronic exposure tests with honey bees and bumblebees establish control mortality criteria at 15 % at 10 days (OECD, 2017a). In our validation of the test, three labs had mortality rates $\leq$10%. Unlike honey bee and bumblebee colonies, which have long activity periods, *Osmia* populations are only active for a couple of months in spring. Depending on the bioclimatic region, *O. bicornis* adults emerge from March to June. Although the timing of emergence can be, within certain limits, adjusted through appropriate management of wintering temperatures (Bosch and Kemp, 2004, 2003), best results are obtained when the tests are conducted during the natural activity time of the population. In our validation of the test, control mortality was related to the timing of the experiments, reaching values $>$ 15% in the two labs working later in the season. There was also a relationship between control mortality and the LDD50 values obtained. Laboratories experiencing control mortality below 15% yielded similar LDD50 values (at 10 days). The LDD50 was lower in Lab 4 (16% control mortality) and could not be calculated in Lab 5 (31% control mortality). Based on these results we propose to perform a preliminary emergence test to determine the optimal flight period of the population (see protocol in Supplementary Information). Our results also allowed us to establish 15% as the control mortality threshold for the validity, in congruence with honey bee tests (OECD, 2017a). Although chronic honey bee tests are conducted over a period of 10 days (OECD, 2017a) we extended survival checks until all bees died. Mean longevity of the control groups was 29.2 days, similar to that of *Osmia* spp. observed in field and semi-field studies (Bosch, 1994; Bosch and Vicens, 2006; Sgolastra et al., 2016; Sugiura and Maeta, 1989; Tepedino and Torchio, 1982), further proving that the test conditions were appropriate for *Osmia*.

We used dimethoate as a positive control, at the same concentration (1 mg/L) proposed for honey bee chronic exposure tests (OECD, 2017a). Mortality in dimethoate-exposed bees exceeded 50% within 10 days, thus meeting the criterion for a positive control outcome. The mean daily dose of dimethoate used in our study was 0.60 μg/bee/day, close to 24-h oral LD50 values for *O. cornuta* (0.66 μg/bee), *O. lignaria* (0.27 μg/bee) and *A. mellifera* (0.15 μg/bee) (Azpiazu et al., 2023a; Ladurner et al., 2005a).

The LDD50 of FPF at 10 days in *O. bicornis* obtained in our study (531.92 ng/bee/day) is 3.4 times lower than that of *A. mellifera* (1830 ng/bee/day; (EFSA et al., 2022) The lowest observed adverse effect dose (256.81 ng/bee/day) is 3 times lower than for *A. mellifera* (NOED = 790 ng/bee/day; EFSA et al., 2022). These results are in agreement with previous oral exposure studies showing that insecticides targeting nAChR, such as neonicotinoids and sulfoxaflor, are more toxic to *Osmia* than to honey bees and bumblebees (Arena and Sgolastra, 2014; Azpiazu et al., 2021; Biddinger et al., 2013; Linguadoca et al., 2022; Sgolastra et al., 2017). Contrasting sensitivity to insecticides among bee species, is commonly attributed to differences in P450 enzymes responsible for xenobiotic detoxification (Beadle et al., 2019; Haas and Nauen, 2021; Hayward et al., 2019; Manjon et al., 2018; Troczka et al., 2019), underscores the need to increase our knowledge base on the comparative sensitivity of different groups of bees. FPF toxicity has been shown to increase synergistically with propiconazole, a sterol biosynthesis inhibiting (SBI) fungicide that inhibits cytochrome P450 (Tosi and Nieh, 2019), highlighting the need to account for multiple exposure to obtain meaningful assessments of the potential risks of realistic pesticide contexts to bee health (Topping et al., 2020).

Syrup consumption was affected by FPF exposure. As previously observed in honey bees (Tosi et al., 2021), high concentrations of FPF had an inhibitory effect on feeding. Similar effects have been reported in studies involving neonicotinoids and sulfoxaflor in honey bees, bumblebees and *Osmia* (Azpiazu et al., 2022, 2019; Laycock et al., 2012; Siviter et al., 2019; Zhu et al., 2017). During the first 10 days of exposure, an increase in syrup consumption at the 4.8 mg/L concentration was observed. The influence of these insecticides on feeding behaviour are known to be dose-dependent, and several studies report stimulatory effects at low doses (Azpiazu et al., 2022; Kessler et al., 2015; Sgolastra et al., 2018). These findings emphasize the need to consider dose-dependency when evaluating the impact of pesticide exposure on bees, as any effects on syrup consumption will directly influence the ingested dose.

Chronic oral exposure experiments allow for the evaluation of cumulative toxicity effects resulting from long-term exposure. We did not find evidence of time-reinforced toxicity of FPF in *O. bicornis*. Our results are consistent across laboratories, except for Lab 5, with high control mortality and lack of a clear dose-response. Our results are congruent with those observed in *A. mellifera* (Tosi et al., 2021), but diverge from studies using other insecticides, such as neonicotinoids and fipronil, which show cumulative toxicity in honey bees and bumblebees (Bommuraj et al., 2021; Mulvey and Cresswell, 2020; Rondeau et al., 2015). Some studies demonstrate that FPF is quickly detoxified and

metabolised by cytochrome P450 (Haas et al., 2021; Hayward et al., 2019).

The first goal of our study was to describe a protocol for long-term pesticide exposure in solitary bees. We showed that control mortality levels as low as 10% can be obtained with *Osmia* and that consistent LDD50 estimates can be obtained when this mortality threshold is met. An adjusted protocol, incorporating elements of a parallel effort to develop a chronic oral test for *Osmia* (Cabrera et al., 2024), is currently being ring-tested by several laboratories. The second goal of our study was to test the toxicity of FPF on *O. bicornis* under chronic oral exposure. FPF is considered a "relatively safe" insecticide for honey bees and bumblebees and its use under label recommendations is allowed during bloom (USEPA, 2014). However, our toxicity results combined with residue levels found in various crops, suggests that FPF could pose a lethal threat to *Osmia* populations under field conditions, a view corroborated by a recent semi-field experiment with *O. lignaria* finding lethal effects in bees exposed to FPF at recommended field rates (Siviter et al., 2024). In view of these results, the use of FPF during crop bloom should be reconsidered. Overall, our research underscores the need for comparative studies to determine whether current safety factors are sufficiently protective of non-*Apis* bees, and a more comprehensive approach to risk assessment including realistic scenarios of long-term exposure. By elucidating the impacts of chronic oral pesticide exposure on *Osmia*, our study contributes to a broader understanding of pollinator health and to the mitigating of pesticide use in agricultural ecosystems.

CRediT authorship contribution statement

Celeste Azpiazu: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fabio Sgolastra:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Alessio Ippolito:** Writing – review & editing, Visualization, Formal analysis. **Sergio Albacete:** Writing – review & editing, Validation, Investigation, Data curation. **Annely Brandt:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Monica Colli:** Writing – review & editing, Investigation, Data curation. **Daniela Grossar:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Lukas Jeker:** Writing – review & editing, Validation, Methodology, Investigation. **Valeria Malagnini:** Writing – review & editing, Validation, Methodology, Investigation. **Gonzalo Sancho:** Writing – review & editing, Validation, Investigation. **Aleksandra Splitt:** Writing – review & editing, Validation, Investigation. **Lars Straub:** Writing – review & editing, Validation, Methodology, Investigation. **Verena Strobl:** Writing – review & editing, Validation, Methodology, Investigation. **Mikolaj Boranski:** Validation, Investigation. **Jacek Jachuła:** Validation, Investigation. **Cátia Martins:** Validation, Investigation. **Piotr Medrzycki:** Methodology, Conceptualization. **Noa Simon-Delso:** Writing – review & editing, Conceptualization. **Simone Tosi:** Writing – review & editing, Methodology. **Jordi Bosch:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization.

Disclaimer

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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.envpol.2024.125129.

Data availability

Data will be made available on request.

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