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Thiamethoxam soil contaminations reduce fertility of soil-dwelling beetles, *Aethina tumida*

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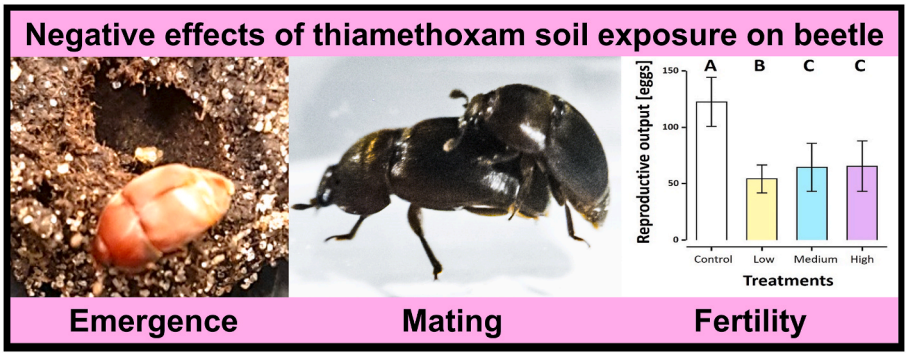
HIGHLIGHTS

- Field relevant soil contaminations of thiamethoxam impair beetle fertility.
- Female adult egg laying rates were reduced by 50% even at the lowest concentration.
- The data reveal a mechanistic explanation for recent declines in insect populations.
- Reductions in soil pollution are urgently required to safeguard insect biodiversity.

GRAPHICAL ABSTRACT



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ABSTRACT

There in increasing evidence for recent global insect declines. This is of major concern as insects play a critical role in ecosystem functionality and human food security. Even though environmental pollutants are known to reduce insect fertility, their potential effects on insect fitness remain poorly understood - especially for soil-dwelling species. Here, we show that fertility of soil-dwelling beetles, *Aethina tumida*, is reduced, on average, by half due to field-realistic neonicotinoid soil contaminations. In the laboratory, pupating beetles were exposed via soil to concentrations of the neonicotinoid thiamethoxam that reflect global pollution of agricultural and natural habitats. Emerged adult phenotypes and reproduction were measured, and even the lowest concentration reported from natural habitats reduced subsequent reproduction by 50%. The data are most likely a conservative estimate as the beetles were only exposed during pupation. Since the tested concentrations reflect ubiquitous soil pollution, the data reveal a plausible mechanism for ongoing insect declines. An immediate reduction in environmental pollutants is urgently required if our aim is to mitigate the prevailing loss of species biodiversity.

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1. Introduction

The rate at which our planet's entomofauna is declining is alarming (Hallmann et al., 2017; Seibold et al., 2019; Wyckhuys et al., 2021) and is almost certain to have a severe impact on the functioning of natural ecosystems and human food security (Daily and Karp, 2015; Klein et al., 2007; Leather, 2018). By providing key ecosystem services, insects play a fundamental role in a variety of ecological processes, including pollination, herbivory and detrivory, as well as providing a food source for higher trophic levels (e.g., birds, fish, and mammals) (Hill, 1997; Yang and Gratton, 2014). Diverse soil biota are essential for facilitating soil formation and improving crop production and their services are estimated at an annual value beyond $2 billion (Pimentel et al., 1997). Soil-dwelling insects are of particular importance as they are essential for ensuring soil fertility and formation (Costanza et al., 1997; Mace et al., 2012) and thereby take an indispensable role not only in terrestrial ecosystems, but also agriculture (Samways, 2005). Unfortunately, soil-dwelling insects and other invertebrates are not exempt from the ongoing global biodiversity losses, highlighted by the recent reports of global declines in beetle species abundance, richness and phylogenetic diversity (Homburg et al., 2019; Sánchez-Bayo and Wyckhuys, 2021).

The drivers underlying these declines are most likely the result of complex interactions among a range of simultaneously acting stressors including habitat loss and fragmentation, anthropogenic pollution, pathogens and invasive species as well as climate change (Sánchez-Bayo and Wyckhuys, 2019; Wagner et al., 2021). However, environmental pollutants, in particular the extensive usage of industrial agrochemicals, such as organophosphates, pyrethroids and carbamates, are a key threat to global biodiversity and so undoubtedly represent one of the greatest existential challenges of the Anthropocene (Chagnon et al., 2015; Habel et al., 2019; Sánchez-Bayo and Wyckhuys, 2019). One particular class of insecticides, the neonicotinoids, has raised considerable concerns, as these are amongst the most widely used insecticides across the globe (Simon-Delso et al., 2015). Whilst the usage of these broad-spectrum insecticides to control pest species is highly effective, they also have inadvertent detrimental lethal as well as sublethal effects on non-target species (; Pisa et al., 2014; Siviter and Muth, 2020). Administered mainly prophylactically as soil and seed treatments but also via spray application (Jeschke et al., 2011), only a small proportion of the water-soluble neonicotinoid is taken up by the crop, with the vast portion (70–95%) ending up in the environment, contaminating soil and water (Mörtl et al., 2020; Wood and Goulson, 2017). The widespread use of neonicotinoids, their high solubility in water and their potential extreme half-lives (i.e., occasionally exceeding several years (Bonmatin et al., 2015)), has not only led to ubiquitous contaminations in agricultural fields, but also areas thought to be neonicotinoid free (e.g., conservation areas (Humann-Guilleminot et al., 2019; Schaafsma et al., 2015)). Subsequently, soil-dwelling organisms are likely to be exposed to chronic levels of neonicotinoids ranging anywhere between <1 to well beyond 200 ng g^{-1} (Bonmatin et al., 2021; Goulson, 2013). Despite clear evidence showing negative effects of neonicotinoid contaminations on soil-dwelling insects (Pisa et al., 2014; Schläppi et al., 2020), as well as the consensus that environmental pollutants can impair fertility (Castellini et al., 2020; Ratcliffe, 1967), their impact on insect fitness remains poorly understood - especially for soil-dwelling species. Data revealing that neonicotinoid soil pollution can interfere with the biological processes of soil-dwelling insects would constitute a novel mechanism underlying their ongoing declines and be of significant relevance for the respective mitigation of this chemical stressor.

The aim of this study was to assess the potential lethal (i.e., emergence success and adult survival) and sublethal (i.e., adult emergence mass, mating behavior and reproductive output (i.e., eggs laid)) effects of field-relevant soil contaminations of a common neonicotinoid insecticide on the small hive beetle, *Aethina tumida*. Therefore, pupating soil-dwelling small hive beetles were exposed via treated soil to the neonicotinoid thiamethoxam at three concentrations. These treatment levels reflect global pollution in managed agricultural and natural habitats, and were used to measure effects on phenotypes and reproduction of the target species. Considering that neonicotinoid exposure can significantly increase mortality as well as impair insect development, fertility and reproduction (Grünewald and Siefert, 2019; Leather, 2018; Strobl et al., 2021; Stuligross and Williams, 2021), we hypothesize that beetles exposed to a neonicotinoid would experience both significant lethal (survival) and sublethal (development, mass and reproduction) detrimental effects from the exposure.

2. Material and methods

2.1. Experimental set-up

All experiments were conducted at the Mississippi State University Apiculture Laboratory in Starkville, Mississippi, USA from January to December 2019 Adult Small Hive Beetles (SHB)s, *A. tumida*, were collected from naturally infested local honey bee colonies, sexed and used to initiate a laboratory rearing (Neumann et al., 2013). In brief, 50-100 adults were housed in plastic containers [4 L] with screened lids, provided with pollen diet (4% protein patty, Global Patties, Butte, Montana; honey; DI in a ratio of 450 g: 50 mL: 30 mL, respectively.), and oviposition sites (two microscope slides taped together with a 1 mm gap between them), and then incubated in complete darkness at 34 °C, 50–68% RH. The containers were checked daily: Slides filled with eggs were transferred to 15 cm petri dishes and the larvae were provided diet *ad libitum* (see pollen diet above) until they reached the post-feeding wandering stage, at which they were then moved onto their respective treatment soils for pupation.

2.2. Soil treatments and exposure

The experiment consisted of four soil treatments (six replicates per treatment); three concentrations of thiamethoxam and a control. A large batch of non-sterilized pupation soil was hand-mixed using organic composted manure (Black Kow®; Oxford, FL) and commercial playground sand (Quikrete® Play Sand, Atlanta, GA) at a volume-to-volume ratio of 2:1, respectively. The physical and chemical properties of the resulting mixture are listed in Supplementary Information (SI) Table 1. The pupation soil was not sterilized and was allowed to dry in a 30 °C incubator to 25% humidity before the neonicotinoid insecticide was added.

Thiamethoxam treatments were prepared using the commercial seed treatment product Cruiser® 5 FS (Syngenta Crop Protection, Inc., Greensboro, NC). Low, medium and high rates of thiamethoxam were made by preparing a pure stock solution (600,000 ng g^{-1} active ingredient) of the product in deionized water and diluting it to three concentrations: 25, 100 and 200 ng g^{-1} active ingredient. These concentrations range within previously reported environmentally realistic soil residue levels of thiamethoxam (Bonmatin et al., 2021; Wang et al., 2020) (see also SI Table 2) The treated soil served as a pupation medium for SHB wandering larvae.

Pupation containers were constructed from 4 L plastic food storage receptacles that were retrofitted with aluminum mesh lids and bottoms to facilitate soil treatments and permit airflow (SI Figs. 1A and 2A). Each container was filled to a pre-marked level with pupation soil (~1.5 kg); the soil was then fully saturated with either an insecticide solution or DI water by submerging the container in a 1 L bath for 24 h. In brief, the aqueous solutions were absorbed via capillary action through the screened bottoms of the containers until soil reached water-holding capacity (Gupta et al., 2008). The mean starting weight of soil in the pupation containers was 1.36 kg ± 0.096 and the mean volume of absorbed solution was 374.7 mL ± 15.2 (mean ± SE). After the pupation containers were soaked, excess solution was drained by elevating the containers for 30 min on a wire rack. The medium was then allowed to equilibrate at room temperature for 24 h before the larvae were added

(Ritchie et al., 2019).

To confirm the vertical distribution of the insecticide in the soil, as well as test for residues in the control group, a core sample from each of the five control and low thiamethoxam treatment replicates was taken immediately before the larvae were placed onto the soil (i.e., day 0). Additionally, to determine the concentration of the insecticides in the soil after adult emergence, two core samples from each treatment group were taken, as well as from each control replicate on day 26 (SI Fig. 2C). The samples were stored at −20 °C until analyzed.

2.3. Soil extraction and analyses

Soil samples were prepared according to a previous protocol with small modifications (Humann-Guilleminot et al., 2019). In brief, samples were homogenized, dried, sieved (2 mm mesh), ground, and weighed (0.5 g) in a 15 mL PP tube. A volume of 4.95 mL of acetonitrile and 50 μL of an internal standard solution (conc. 500 ng mL^{-1} of thiamethoxam-d3 and clothianidin-d3 (primary metabolite of thiamethoxam), and 200 ng ml^{-1} of permethrin-d5) were added and the samples were extracted overnight on a vertical rotation shaker at 60 rpm. The use of isotopically labelled internal standards allows to correct for the overall analytical variation occurring during the measurement process. Permethrin, a synthetic organic pyrethroid, is a globally popular insecticide for agricultural purposes and contaminations are frequently detected in soils (Ensminger et al., 2013; Li et al., 2017; Tang et al., 2018). To account for the potential further insecticide contaminations, we additionally tested for permethrin in all the control samples. The tubes were centrifuged at 4000 g and as much supernatant as possible was pipetted into new 15 mL PP tubes containing a mixture of salts and 5 mL of water for QuEChERS extraction. Samples were further purified by dispersive solid-phase extraction using a mixture of MgSO4, C18 and PSA sorbents. The extracted samples were injected without any dilution for permethrin quantification, or diluted 5-fold with water for thiamethoxam and clothianidin measurements. To quantify thiamethoxam and clothianidin in the treatment soils after the adults emerged, two core soil samples of each treatment group (i.e., low, medium and high) were analyzed using Ultra-high pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) (Waters Acquity UPLC I-Class coupled to TQ-XS triple quadrupole) as previously described (Humann-Guilleminot et al., 2019; Kammoun et al., 2019). In addition, five samples of control soil were tested to identify any trace of thiamethoxam and clothianidin in the soil (SI Table 3). Permethrin was also analyzed by UHPLC-MS/MS but using a slightly different method from that used for neonicotinoids. An Acquity UPLC BEH C18 column (50 × 2.1 mm i.d.) at 45 °C was used for the separation. Mobile phases were milli-Q water supplemented with formic acid 0.05% and ammonium formate 1 mM, and LC-MS grade acetonitrile containing 0.05% formic acid. A gradient from 65% to 100% acetonitrile was performed in 2 min, followed by a wash at 100% for 2 min. The flow rate was 0.4 mL min^{-1}. The injection volume was 5 μL. The mass spectrometer was operated in positive electrospray ionization, using a capillary voltage of 4 kV, a desolvation temperature of 400 °C, a desolvation gas flow of 1000 L h^{-1} and a cone gas flow of 350 L h^{-1}. The StepWave was set to soft transmission. MRM quantitative and qualitative transitions for permethrin were 408 > 183 and 410 > 183, respectively. For permethrin-d5, a single transition was used (413 > 188). Under these conditions, the two isomers of permethrin were baseline separated and eluted at 1.76 and 1.85 min, respectively. The entire system was controlled by Masslynx 4.2 (Waters) and peak integration was done in the software TargetLynx (Waters). A 5-point calibration curve ranging from 0.2 to 50 ng mL^{-1} was used for quantification. The limit of quantification for permethrin was 0.2 ng mL^{-1} or 2 ng g^{-1} of dry soil and 0.0019 and 0.0016 ng g^{-1} for thiamethoxam and clothianidin in dry soil, respectively. Samples that revealed concentrations below the limits of quantification (<LOQ) were set to zero and accounted for when calculating the arithmetic mean of the soil contamination in each treatment.

2.4. SHB emergence success and emergence mass

Once the soil treatment groups were prepared for each replicate, one hundred ($N = 100$) post-feeding wandering SHB larvae were randomly

Table 1
Statistical summary of variables measured on small hive beetles, *Aethina tumida*, exposed to increasing concentrations of thiamethoxam (Low [25 ng g^{-1}], Medium [100 ng g^{-1}], and High [200 ng g^{-1}] contaminated soil. For each variable the sample size, mean, standard error (Std. Err.) as well as the lower and upper 95% confidence intervals (C.I.) are reported.

Variables	Sex	Treatments	Sample sizes	Mean	Std. Err.	Lower 95% C.I.	Upper 95% C.I.
Emergence success [%]	Both	Control	300	97.7	0.009	95.9	99.3
		Low	300	95.4	0.012	93.1	97.7
		Medium	300	90.3	0.017	86.9	93.7
		High	300	89.7	0.018	86.2	93.1
Emergence time [d]	Both	Control	292	17.75	0.086	17.6	17.9
		Low	289	17.86	0.092	17.7	18.1
		Medium	261	17.74	0.091	17.6	17.9
		High	268	17.25	0.083	17.1	17.4
Emergence mass [mg]	Male	Control	91	1.53	0.013	1.51	1.56
		Low	82	1.43	0.019	1.39	1.47
		Medium	82	1.41	0.018	1.37	1.45
		High	71	1.44	0.014	1.41	1.47
	Female	Control	119	1.63	0.012	1.61	1.66
		Low	131	1.52	0.014	1.49	1.55
		Medium	89	1.53	0.018	1.49	1.57
		High	109	1.52	0.012	1.49	1.54
Oviposition activity [%]	Female	Control	50	74	0.063	61.4	86.6
		Low	50	38	0.069	24.1	51.9
		Medium	50	48	0.071	33.6	62.3
		High	50	58	0.071	43.8	72.2
Onset of oviposition [d]	Female	Control	37	3.97	0.468	3.1	4.9
		Low	19	6.21	0.609	4.9	7.5
		Medium	24	6.33	0.633	5.1	7.6
		High	29	7.01	0.631	5.8	8.4
Reproductive output [eggs]	Female	Control	37	122	21	78	166
		Low	19	54	12	28	80
		Medium	24	64	21	20	108
		High	29	65	22	20	111

assigned to each of the four groups and incubated at 30 °C in complete darkness. Based on previous adult beetle emergence under the given conditions (Neumann et al., 2016), daily observations were made after day 16 to ensure that all successfully emerging beetles were obtained. Individuals that did not emerge by day 26 were considered to have failed to complete development to adult. To confirm that no living beetles were overlooked, the entire soil content of each pupation container was sieved on day 26 (U.S.A. Standard Test Sieve series [4.0 mm/1.7 mm/1.0 mm]). The sieving of the soil revealed that no living adults remained in the soil. Adults emerging from the soil were collected, weighed to the nearest 0.01 g (Mettler Toledo, Model #: AL204, Columbus, OH, USA), sexed (Neumann et al., 2013) and separated into respective 236 mL 'male' and 'female' ventilated containers (Uline, Wisconsin, United States of America) (SI Fig. 1B), dated and provisioned with pollen diet (see above) and water. Emergence success [%] was determined by dividing the total number of emerged individuals by 100. Beetles were grouped by emergence date and kept on pollen diet and water for one week to allow them to reach sexual maturity (Neumann et al., 2013; Papach et al., 2021) (SI Fig. 2B).

2.5. Effects of exposure on small hive beetle mating success and reproductive output

Time of oviposition and total number of eggs laid were estimated as tokens of fitness. For each replicate, mating pairs of sexually mature adult SHB from the same treatment group were established. Beetle couples were introduced to small perforated disposable petri dishes containing a moist filter paper (Fisherbrand, 50 × 11 mm petri dishes; Fisherbrand P5 Qualitative filter paper, 45 mm; Rochester, NY, USA; SI Fig. 3A) and 10 μL pollen diet. Daily recordings were made of eggs laid within the petri dishes; subsequently, all eggs were carefully removed to facilitate counting on the following day (SI Fig. 3C). Onset of oviposition, measured when an egg was first laid within the petri dish, was monitored for 10 days. Reproductive output was measured as the total number of eggs laid within this period. Females that did not lay any eggs within 10 days were considered unsuccessfully mated and their oviposition activity [eggs] was recorded as '0'. Filter papers were replaced every second day, unless they had eggs or soiled with feces, in which case they were replaced daily. Lastly, the survival of the adult beetles was recorded daily.

2.6. Data analyses

All statistical tests were performed using STATA16, while statistical figures were created using NCSS 20. Data were tested for normality by using a Shapiro-Wilk test. Homogeneity of variances was confirmed by visually inspecting the residual plots as well as using the Levene's *F*-test with the function *sdtest*. Linear (regression) mixed-effects models (LMMs) were applied using the function *regress* to assess potential relationships among explanatory variables and the dependent variables. Multilevel generalized logistic or linear regression models (GLMMs) with random intercepts were fitted STATA16. Terms were defined as follows: individual SHBs were considered independent units; treatments (neonicotinoid vs. control) and sex were included as the explanatory (fixed) terms; and replicate, body mass and emergence duration were incorporated as random effects whenever applicable (Leckie, 2010). A stepwise backward elimination approach was applied to determine the model of best fit for each multiple regression analysis. Best fit models were chosen by comparing every multi-level model with its single-level model counterpart. Both a likelihood ratio (LR) test as well as the Akaike information criterion (AIC) and the Bayesian information criterion (BIC) were used with the functions *lrtest* and *estat ic,* respectively (Sribney and StataCorp, 2005). Post-hoc comparisons for all variables among treatment groups were conducted using a multiple pairwise comparisons test (Bonferroni Multiple Comparisons Test (*bmct*)), defined by the function *mcompare(bonferroni)*. Whenever appropriate, either the arithmetic means of non-transformed values ± the standard error (SE) or the 95% confidence intervals (CI) are given in the text.

Logistic GLMMs were applied to test for treatment differences for the binary outcome variables Emergence success [%] and Oviposition activity [%] using the function *melogit*. The conditional distribution of the regression given the random effects was considered to be Bernoulli. The sex-ratio of emerged males to females was recorded as a score; subsequently, an ordered logistic model was applied using the function *meologit*. Emergence time in days [d], Emergence mass [mg], and Onset of oviposition [d] were modeled with linear GLMMs of the Gaussian or Gamma family (depending on the analysis of residuals) using the function *meglm*. Counter transforming the outcome variables showed that the Gamma family provided good fits (normality of the residuals). The Reproductive output [eggs] data were non-parametrically distributed (Shapiro-Wilk, $p < 0.001$), as several females laid no eggs ($N = 120$), causing a zero inflation effect. Therefore, a zero-inflated Poisson model was applied with Treatment and Oviposition activity as fixed effects to analyze the excess zero counts. This model adequately captures excess zeros by calculating incidence rate ratios separately for the zero inflation in both the Treatment and Oviposition effects. The zero inflation was significant for both Treatment and Oviposition activity (both p's < 0.001), thus the model generated incidence rate ratios for the treatment and oviposition terms without the excess zeros. Survival times for individuals were fitted using the function *mestreg* for multilevel survival models with a Weibull distribution (Cleves, 1999).

3. Results

Soil residue analyses: The UHPLC-MS/MS analyses of the control samples revealed 3.76 ± 2.14 ng g^{-1} and 1.81 ± 1.15 ng g^{-1} of thiamethoxam and clothianidin, respectively (mean ± SE). Further, the 25 ng g^{-1} thiamethoxam treatment samples that were analyzed directly following insecticide application revealed an average thiamethoxam residue of 27.63 ± 0.27 and 5.05 ± 2.03 clothianidin (mean ± SE); therefore, confirming the effectiveness of the applied soil exposure method and presence of the insecticide. After the beetles had emerged, the controls as well as the low, medium, and high neonicotinoid exposed treatments revealed thiamethoxam residues of 0.33 ± 0.25, 14.25 ± 2.44, 81.64 ± 10.48 and 159.02 ± 33.27 ng g^{-1}, respectively [mean ± SE]. In addition, one control sample revealed traces of the insecticide permethrin (2.984 ng g^{-1}). All results of the pooled soil samples taken at the beginning and the end of the experiment can be found in SI Table 3.

Emergence success: The data revealed a significant negative effect of thiamethoxam exposure on the emergence success of adult SHBs (Wald $X^2_{(2,\ 1203)} = 7.04$, $z = -3.53$, $p < 0.001$). Total adult emergence ($N = 300$ per treatment) for the control, low, medium, and high groups was 293, 281, 271, and 274, respectively. The low thiamethoxam treatment did not significantly differ from the controls (*bmct*; $p = 0.80$; Fig. 1A; Table 1); both showed success rates ranging from 95.4 ± 0.01 to 97.7 ± 0.01 (mean ± SE [%]). However, both medium and high treatment groups significantly differed from the controls and low treatment groups (bmct; both p's < 0.05; Fig. 1A), resulting in a reduction of 7.7% compared to the controls. The medium (90.3 ± 0.02) and high (89.7 ± 0.02) thiamethoxam treatment groups did not significantly differ from each other (*bmct*; $p = 1.0$; mean ± SE [%]).

Sex-ratio: Thiamethoxam exposure revealed no significant effect on the sex-ratio of SHBs (Wald $X^2_{(2,\ 9)} = 0.85$, $z = 0.19$, $p = 0.85$). No significant differences were observed among the treatment groups (*Wald* $X^2 = 1.60$, $p = 0.66$); with the average sex ratio across of all treatments ranging between 1.03 ± 0.08 and 1.25 ± 0.13 in favor of females.

Emergence time: Both emergence mass and emergence time significantly negatively correlated with neonicotinoid exposure (Wald $X^2_{(3,\ 781)} = 71.68$, both z's < 6.04, p's < 0.001). Subsequently, increased thiamethoxam exposure led to a reduced emergence time (Fig. 2A),

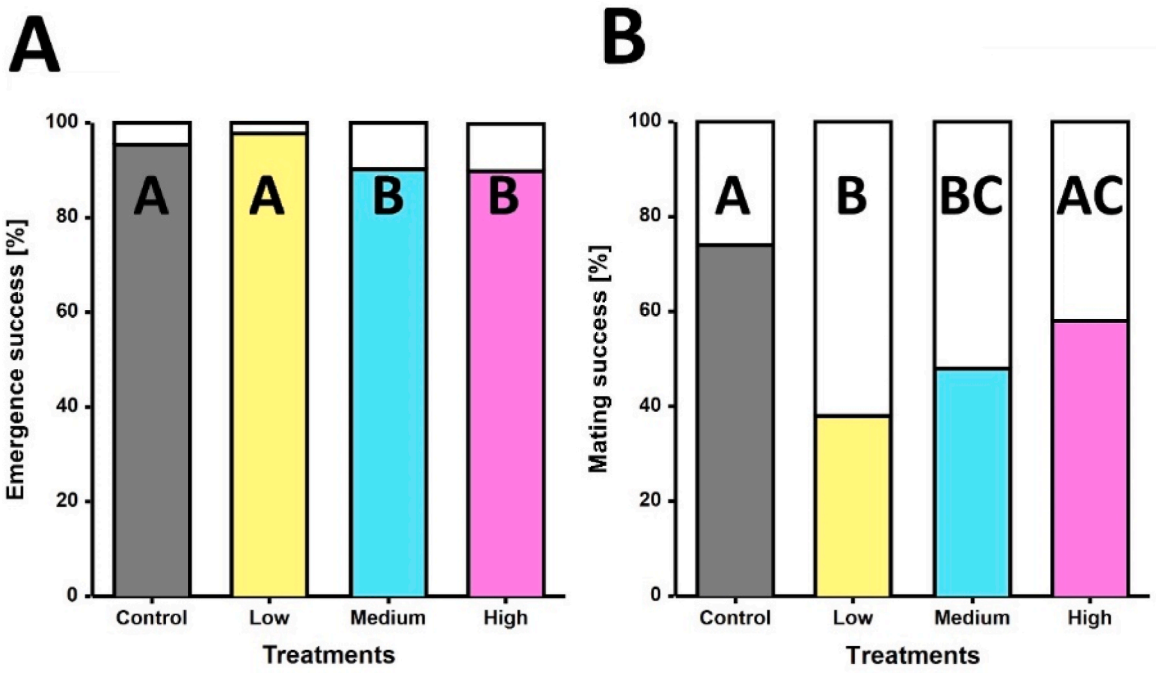

Fig. 1. Small hive beetles,*Aethina tumida*, were exposed to increasing concentrations of thiamethoxam (TMX) [25 (Low), 100 (Medium) and 200 (High) ng g^{-1}] contaminated soil. Potential effects on the sublethal parameters Emergence success [%] and Emergence success [%] were assessed. (A) The emergence success [%] shows the proportion of larvae that survived the exposure period and developed to an emerging adult beetle. **(B)** Mating success was determined during the mating trials, wherein females that laid at least one egg were considered successfully mated and reproductive active. Color shaded areas in the bar charts represent proportion of beetles that emerged and mated successfully, whereas white shaded areas represent the proportion of beetles that did not emerge or mate successfully. All statistical analyses were performed using generalize linear mixed models (GLMMs) and significant differences ($p < 0.05$) between treatment groups and the controls are indicated by letters (**A**, **B** and **C**).

which led to an increased emergence time (SI Fig. 4). Control (17.75 ± 0.05) emergence time did not significantly differ from the low (17.86 ± 0.09) and medium (17.75 ± 0.09) thiamethoxam treatments (*bmct*; both p's > 0.55; Fig. 2A; mean ± S.E. [d]). However, all treatment groups significantly differed from the high exposure group (bmct; all p's < 0.022; Fig. 2A), with the high treatment group showing the shortest emergence time (17.25 ± 0.08; mean ± S.E. [d]; Fig. 2A).

Emergence mass: Sex, thiamethoxam exposure, as well as emergence time, all revealed a significant effect on emergence mass (Wald $X^2_{(3, 781)} = 126.57$, all z's < −7.45, p's < 0.001), with males being lighter than

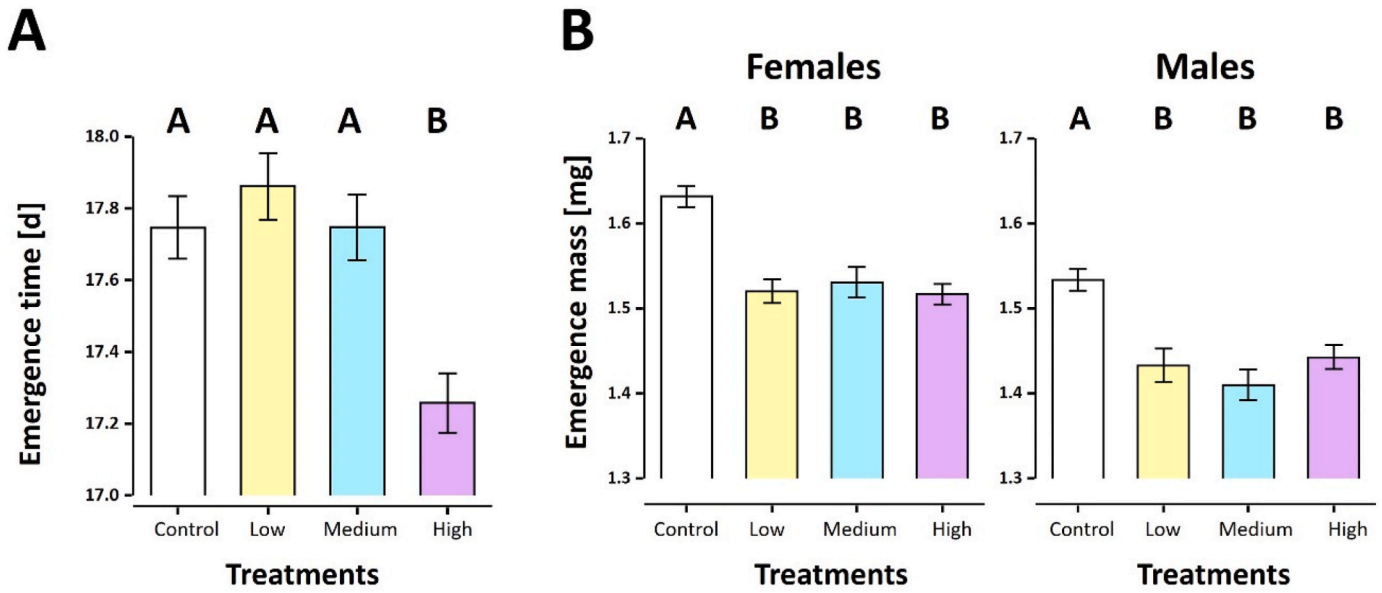

Fig. 2. - Small hive beetles,*Aethina tumida*, were exposed to increasing concentrations of thiamethoxam (TMX) [25 (Low), 100 (Medium) and 200 (High) ng g^{-1}] contaminated soil. Potential effects on the sublethal parameters Emergence time [d] and Emergence mass [mg] were assessed. (A) Emergence time was significantly reduced in the High treatment group, whereas the Low and Medium did not significantly differ. **(B)** Emergence mass was significantly affected by the insecticide exposure, wherein all treatment groups differed from the controls. This result was observed for both sexes. Significant differences ($p < 0.001$) among treatment groups are indicated by the alphabetical letters (**A** and **B**). Bar charts show the mean and the corrected standard errors.

females. Across all treatment groups, males were significantly lighter than their female counterparts (*bmtc,* all p's < 0.05; Fig. 2B). Further, male and female control groups differed from their respective insecticide treatment groups (*bmtc,* all p's < 0.001), with males being 6–7% heavier and females being 6–11% heavier in the control groups (Fig. 2B). However, for both sexes no significant differences were observed among thiamethoxam treatment groups (*bmtc,* all p's $= 1.00$). Irrespective of sex and treatment group, a significant negative correlation was revealed between emergence time and emergence mass ($F_{(2,\ 785)} = 121.62$, $R^2_{adjusted} = 0.274$, $p < 0.001$), with lighter beetles having longer emergence times (SI Fig. 4).

Adult mortality: Thiamethoxam exposure had no significant effect on survival for either sex (*bmct*, $p > 0.16$), where overall female and male mortality ranged between 2-6% and 10–16%, respectively. Irrespective of the treatment, male mortality was significantly higher compared to female mortality (*mestreg*; $p < 0.001$).

Oviposition: The data revealed that thiamethoxam exposure had a significant negative effect on the oviposition activity of SHBs (*Wald* $X^2_{(2,\ 197)} = 7.22$, $p < 0.007$; Fig. 1B). The low (38 ± 24–52%) and medium (48 ± 34–62%) treatment groups both significantly differed from the controls (74 ± 61–86%) (*bmct;*all p's < 0.015; mean ± 95% CI [%]; Fig. 1B); resulting in reduction in mating success (i.e., oviposition activity) of 49% and 35%, respectively. The high (58 ± 43–72%) treatment did not significantly differ from the control treatment group (bmct; all$p$'s>0.05; mean ± 95% CI [%]; Fig. 1B), despite the reduction in mating success of 22%. The high treatment group did not significantly differ from the medium ($p = 0.31$), yet did significantly differ from the low treatment ($p = 0.45$; Fig. 1B). Thiamethoxam exposure had a significant negative effect on the onset of oviposition (*Wald* $X^2_{(2,\ 105)} = 10.88$, $p < 0.001$; Fig. 3A); however the age difference within mating pairs did not reveal a significant effect ($p > 0.16$). Control females (day 4 ± 0.5) started significantly earlier compared to the low (day 6 ± 0.5), medium (day 6 ± 0.5) and high (day 7 ± 0.5) thiamethoxam treatment groups (bmct; $p < 0.013$; Fig. 3A; mean ± SE [d]); yet no significant differences were observed among the exposed treatment groups (*bmct*; all p's $= 1.0$; Fig. 3A). In comparison to the controls, the delay in onset of oviposition for the thiamethoxam exposed females ranged between 2 and 3 days.

Reproductive output: While thiamethoxam exposure had a significant negative effect on the reproductive output of females (*Wald* $X^2_{(3,\ 105)} = 2.72$, $p < 0.006$), the age difference within mating pairs revealed no significant effect ($p = 0.49$). Further, a significant negative correlation was determined between onset of oviposition and reproductive output across all treatment groups ($F_{(2,\ 247)} = 13.71$, $R^2_{adjusted} = 0.1$, $p < 0.001$; Fig. 3B), where delayed oviposition resulted in fewer eggs after the 10 day observation period. Females from the control group produced the most eggs within the first 10 days (122 ± 21), and significantly differed from the remaining treatment groups (*bmct; p* < 0.001; Fig. 3B; mean ± SE [eggs]). While no significant difference was observed between medium (64 ± 21) and high (65 ± 22) exposure treatments (*bmct*; $p = 1.0$; mean ± SE [eggs]), these two groups significantly differed from the low (54 ± 12) exposure treatment (*bmct*; both p's < 0.001; Fig. 3B; mean ± SE [eggs]). In comparison to the controls, the reduction in eggs laid by the low, medium and high thiamethoxam treatment groups was 55%, 48%, and 47%, respectively; this resulted in an average reduction of 50%.

4. Discussion

The data show that neonicotinoid soil pollution at concentrations reported from agricultural and natural habitats across the globe (Bonmatin et al., 2015; Zhou et al., 2021), can elicit a diverse array of lethal (i.e., adult emergence success) and sublethal (e.g., emergence time and body mass) effects on small hive beetles. Of particular concern were the novel data revealing 50% reductions on reproduction in exposed beetles.

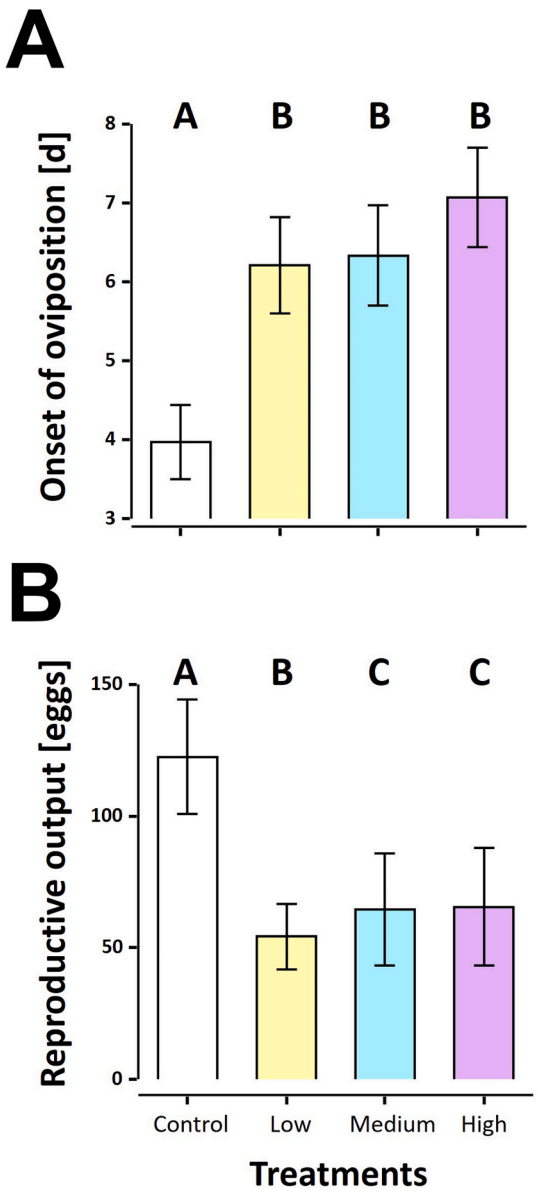

Fig. 3. - Small hive beetles,*Aethina tumida*, were exposed to increasing concentrations of thiamethoxam (TMX) [25 (Low), 100 (Medium) and 200 (High) ng g^{-1}] contaminated soil. Effects on the reproductive parameters onset of oviposition [d] and reproductive output [number of eggs] were assessed. (A) All TMX treatment groups significantly differed from the controls, with the High treatment requiring the longest time for the onset of oviposition. **(B)** All treatment groups significantly differed from the controls. Low revealed the lowest production of eggs and significantly differed from the Medium and High treatments. Significant differences ($p < 0.001$) among treatment groups are indicated by the alphabetical letters (**A**, **B**, **C**). Bar charts show the mean and the corrected standard errors.

Therefore, the data strongly suggest that ubiquitous soil pollution interfering with fertility constitutes one plausible mechanism underlying the ongoing insect declines. Taken together with similar reports from other insect species (Pisa et al., 2014), and in light of their key role for both ecosystem functioning and human food security (Losely and Vaughan, 2006), it appears evident that policy-makers should strive to prevent further inadvertent soil contaminations by implementing more rigorous restrictions on the usage of industrial agrochemicals – in particular the prophylactic application. Sound policies for sustainable agriculture and effective conservation programs are required to reduce such environmental pollution and thereby protect biodiversity and

human food security before it is too late for meaningful action.

Despite using commercial organic soil, the control treatments revealed residues of thiamethoxam as well as its primary metabolite, clothianidin. Considering the increasing global usage of neonicotinoids (Goulson et al., 2018), as well as their water solubility and subsequent high soil mobility (Hladik et al., 2018), such contaminations are almost inevitable and vastly expected in any terrestrial and aquatic ecosystems (Gunstone et al., 2021; Mörtl et al., 2020). Depending on cropping history, rainfall and soil properties, the detected quantities may vary considerably (Goulson, 2013). Contaminations similar to those observed in our controls have been revealed in soils from non-agricultural sites, including nature conservation areas, urban parks and playgrounds (Linhart et al., 2021; Schaafsma et al., 2015; Zhou et al., 2021). Of particular concern are the long half-lives of these chemicals (reportedly >6′000 days) resulting in neonicotinoid residue detections 20 years after the last application (Riedo et al., 2021). Therefore, obtaining non-contaminated soil products for agricultural, private or experimental purposes may be extremely difficult. In any case, the detection of trace contaminations even in our controls highlights that soil-dwelling organisms are confronted with chronic exposure to fluctuating concentrations and mixtures of neonicotinoids worldwide. As expected, our soil samples revealed trace amounts of the insecticide permethrin at levels similar to those previously reported (Li et al., 2017; Tang et al., 2018), thereby confirming that soil-dwelling insects are likely exposed to a cocktail of xenobiotics (Tang and Maggi, 2021). Further, as we used the commercial product Cruiser®, we cannot exclude the possibility that co-formulants or adjuvants that are known to have distinct negative effects (Straw et al., 2022) and may so have contributed to the observed findings. Nevertheless, we are confident that the experimental design and results are robust, as the same soil was applied to all treatment groups. Further, the residue levels detected in our controls are several orders of magnitude lower than the recommended commercial application rates and significantly lower than residue levels commonly detected in soils recently treated with the same insecticides (Bonmatin et al., 2021). Lastly, the use of the commercial product, rather than the active ingredient alone, reflects a more environmentally realistic scenario when investigating the potential impact of frequently applied insecticides on soil-dwelling insects.

The data are the first to reveal a dose-dependent effect of field-realistic neonicotinoid soil contamination on beetle pupation success, thereby confirming reports from other insect species (Barmentlo et al., 2021; Heneberg et al., 2020). While the lowest tested concentration (25 ng g^{-1}) had no significant effect, the medium (100 ng g^{-1}) and high (200 ng g^{-1}) treatment groups reduced successful small hive beetle pupation by 8%. This indicates an additional stressor (besides factors such as nutrition and/or temperature (Scharf et al., 2015)) to populations in agricultural areas displaying similar contamination levels (Bonmatin et al., 2021). Similar detrimental effects have been shown in arthropods across several taxonomic groups (Sánchez-Bayo et al., 2016), including soil-dwelling species such as earthworms (*Eisenia andrei*), springtails (*Folsomia candida*) and mites (*Oppia nitens*) (Ritchie et al., 2019; van Loon et al., 2022). However, in contrast to small hive beetles, these soil-dwelling organisms experienced long-term chronic exposure, as they spend their entire life in contact with contaminated soil, and in the case of earthworms, even ingest contaminated soil. Small hive beetles are only exposed as post-feeding wandering larvae and during pupation. This is, however, a pivotal and highly vulnerable phase in the life cycle of any holometabolic insect species as they undergo metamorphosis, which is strongly regulated by the endocrine system (Truman and Riddiford, 2019). Neonicotinoids are known to act as endocrine disrupting chemicals (Baines et al., 2017), thus likely impairing essential genetic and physiological mechanisms responsible for regulating developmental stability during metamorphosis. Indeed, our data are in line with previous studies showing that thiamethoxam exposure during insect metamorphosis can manifest in altered development, including hindered pupation and metamorphosis (Friedli et al., 2020; Heneberg et al., 2020; Tavares et al., 2017).

Successfully emerged beetles from the neonicotinoid treatments revealed various sublethal effects supporting the assumption of increased stress during development. While the emergence duration across all treatments was well within the range of previously reported pupation times (Neumann et al., 2016), the beetles exposed to the highest concentration emerged significantly faster than the controls. Similar effects on developmental duration are well-documented in bees and are likely attributed to neonicotinoids disturbing the cholinergic system, which is essential for development (Grünewald and Siefert, 2019). Considering that the negative effects on emergence rate and developmental time were only observed in the medium and high treatment group, one could infer that the low neonicotinoid soil pollution (i.e., <27.6 ng g^{-1}) appears safe for beetles.

However, irrespective of concentrations all exposed beetles revealed reduced body mass upon emergence compared to the controls, which may be due to metabolic dysregulation and/or altered glycolytic pathways (Cook, 2019; Wang et al., 2018). Alternatively, and not mutually exclusive, the energetically demanding detoxification pathways may come into play (Castañeda et al., 2009; Evans et al., 2018). Beetles may have invested fat body reserves to ensure that these pathways could be maintained, thus causing a reduced emergence mass. Ultimately, the reallocation of resources to enable detoxification and ensure survival are likely to have come at the expense of other physiological functions (e.g., reproductive traits) at a later life-stage (Flatt and Heyland, 2011; Zera and Harshman, 2001). Irrespective of the underlying mechanisms, body mass is an important fitness proxy in insects and is strongly correlated with reproductive output (Honěk, 1993; Leather, 2018). Therefore, the observed reduced body mass across all treatment groups may act as an early indication for compromised reproduction and reduced fitness.

To our knowledge, the data are the first to reveal a significant negative impact of environmentally realistic thiamethoxam soil contamination on beetle reproduction. Indeed, even the lowest concentration representative of natural habitats reduced reproduction by half. Across treatments, the proportion of females starting oviposition was lower than in the controls. Interestingly, the effect was strongest in the low concentration and not significant in the highest. While the most parsimonious explanation may simply be due to an erratic result or natural variability amongst the species, it may also reflect a biphasic dose response which is well-known from other systems (Calabrese, 2005). Yet, additional data are required to shed light on the exact underlying reason of this finding. The time required to start oviposition was significantly longer across all treatments compared to the controls. Such a delayed onset of oviposition almost certainly reduces reproductive output because these parasitic beetles are facing intra- and interspecific competition in the short time window when protein rich food is not protected by the insect host (typically honey bees) (Neumann et al., 2016). Most importantly, a striking impact of soil pollution was revealed on those females laying eggs, where even the lowest tested concentration reduced the number of eggs laid by 55%. Also, the medium and high concentration revealed reduced egg production by 47–48%, which were all significantly different from the control reproduction. Possible reasons could be impaired mating success as well as compromised reproductive physiology due to neonicotinoid exposure as known from other species (Straub et al., 2016, 2021; Strobl et al., 2021; Williams et al., 2015). Such trade-offs between survival and fitness parameters are well-known (Schwenke et al., 2016) and may be due to costly detoxification at the expense of other traits (Flatt and Heyland, 2011).

Irrespective of the mechanism underlying the reduced reproduction and the actual fate of the larvae, pupae, and later emerging adults, a reduction of half of the eggs being laid will inevitably have a drastic impact on any insect population. Whatever stressor is to come later will even further reduce the number of sexually reproductive adults. Taken together with the 8% less emerging adult beetles, the data suggest a strong effect of neonicotinoid soil pollution at the population level. In the field, neonicotinoids are seldom the only chemicals detected in soil

samples; rather, cocktails of different pollutants are found (de Souza Machado et al., 2018; Geissen et al., 2021; Silva et al., 2019), which may increase overall arthropod toxicity (Siviter et al., 2021). Indeed, even today residues of DDT can be found in Antarctic penguins (Bravo et al., 2019; Sladen et al., 1966). It seems as if we are facing a déjà-vu with the entire situation being very similar to the DDT discussion held in the last century (Grier, 1982). Furthermore, habitat destruction, climate change, pests and pathogens, as well as invasive species are likely to act in concert with such pollutants, in particular the clear impact of the neonicotinoid soil pollution demonstrated here. The data are therefore likely a conservative estimate of the actual impact of global soil pollution on insect populations in the field. Since neonicotinoids are ubiquitous (Hladik et al., 2018; Morrissey et al., 2015), we have reasons to believe that such soil pollution constitutes a key driver for the globally observed insect declines.

5. Conclusion

In conclusion, the data provide clear evidence that neonicotinoids can adversely impact soil-dwelling beetle fertility, which will inevitably have adverse effects on their individual fitness and ultimately at the population level. Further studies are required to determine whether these findings hold true for a broad range of xenobiotics both individually and in combination, and whether these effects can be confirmed across other soil-dwelling insect taxa. Irrespectively, it appears evident that an immediate reduction in environmental pollutants is urgently required if our aim is to mitigate the prevailing loss of biodiversity. Action must be taken to ideally overhaul the prophylactic usage of pesticides in agricultural systems and if applied prophylactically they should be subjected to more rigorous regulatory restrictions. Further, we urge risk assessments to determine the ecological effects of agrochemicals on fitness across a wider range of species (Straub et al., 2020). Policymakers should strive to reinforce the implementation of multi-tiered integrated pest management (IPM), where chemical applications are the last resort (Wyckhuys et al., 2021). Lastly, while their potential to meet current and future food demands remains largely overlooked, drawing on indigenous soil knowledge is a promising approach on both a practical and theoretical level for fostering sustainable agricultural development (Kurashima et al., 2019; Pawluk et al., 1992).

Author statement

L.S and A.B.S. designed the experiment. L.S., A.B.S., E.J.J., A.J.V-M., G.G. collected the data; L.S., J.H., and P.N. provided materials and reagents; L.S. designed the statistical analysis; L.S., A.B.S., and P.N. analyzed the data and wrote the manuscript. All authors discussed the results and contributed to editing and approving the manuscript.

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Data accessibility and availability

The complete raw data can be found at the Dryad repository. See the following link: https://doi.org/10.5061/dryad.s4mw6m9b7.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lars Straub reports financial support was provided by Vinetum Foundation.

Data availability

The complete raw data can be found at the Dryad repository. See: https://doi.org/10.5061/dryad.s4mw6m9b7.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.139648.

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