Exposure of Insects to Current-Use Pesticides in Agricultural Landscapes: Temporal Occurrence, Spatial Distribution, and Implications for Risk Assessment

by

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Abbreviation List

CUP	Current-use pesticide
DT_{50}	Dissipation time, half-life
FVI	Flower-visiting insects
HQ	Hazard Quotient
LOD	Limit of Detection
LOQ	Limit of Quantification
MITAS	MIxture Toxicity of Application Spray series (model)
MRQ	Mixture risk quotient
MQL	Method Quantification Limit
NOEC	No observed effect concentration
PD	Prothioconazole-desthio
RQ	Risk Quotient

Thesis Summary

English Abstract

The global insect decline has been linked, among other factors, to the use of pesticides. Although pesticides are tested during authorisation processes and evaluated for their environmental safety, scientific studies document pesticide residues and effects beyond the scenarios considered in regulatory assessments. This dissertation investigates the exposure pathways of insects to pesticides in agricultural landscapes. For this purpose, multiresidue methods were developed to analyse current-use pesticides (CUPs) in environmental compartments relevant to insects and were subsequently applied in several field studies to investigate their spatial and temporal dynamics.

Substance-specific drift was detected even under agricultural best practice conditions, including the use of 90% drift-reducing technology and best-case weather conditions without wind. A high-resolution year-round monitoring approach demonstrated a chronic presence of CUP mixtures in soils. It pronounced seasonal dynamics in vegetation, with CUP numbers peaking during periods with high insect activity. CUP mixtures were detected not only within treated fields and adjacent habitats, but also at distances of up to 30 km. Based on these data, high-resolution landscape-level predictions of CUP occurrence were generated. The results showed that complex CUP mixtures occur chronically and ubiquitously in agricultural fields and non-target areas, including nature conservation areas. High numbers of CUPs were also detected in environmental matrices, which are particularly relevant for flower-visiting insects. Differences between pollen samples of wild bees and honey bees indicated both distinct exposure and high temporal variability in exposure. CUP residues were also detected in newly hatched wild bees. Risk calculations based on measured soil concentrations indicated risks for non-target organisms. Overall, the results of this dissertation indicated that realistic exposure of insects is not sufficiently addressed in the current authorisation procedure and that risks arising from chronic exposure to CUP mixtures cannot yet be adequately assessed.

Zusammenfassung

Der globale Rückgang der Insekten wird unter anderem mit der Nutzung von Pestiziden in Zusammenhang gebracht. Obwohl Pestizide im Zuge ihrer Zulassung getestet und hinsichtlich ihrer Umweltverträglichkeit bewertet werden, dokumentieren wissenschaftliche Studien Pestizidrückstände und Effekte jenseits der im Zulassungsverfahren berücksichtigten Szenarien. Im Mittelpunkt dieser Dissertation steht die Untersuchung der Expositionspfade von Insekten gegenüber Pestiziden in landwirtschaftlich geprägten Landschaften. Hierfür wurden Multiresiduen-Methoden zur Analyse von Pestiziden in für Insekten relevante Umweltkompartimente entwickelt und anschließend in verschiedenen Feldstudien eingesetzt, um räumliche und zeitliche Dynamiken von Pestizidrückständen zu untersuchen.

Substanzspezifische Drift wurde selbst unter guter landwirtschaftlicher Praxis mit 90% mindernder Spritztechnik und bei besten Witterungsbedingungen ohne Wind nachgewiesen. Ein zeitlich hochauflösender Monitoring-Ansatz über ein Jahr hinweg zeigt eine chronische Belastung der Böden mit komplexen Pestizidmischungen sowie ausgeprägte saisonale Dynamiken in der Vegetation mit Spitzenwerten bei hoher Insektenaktivität. Pestizidmischungen wurden nicht nur innerhalb der behandelten Felder und angrenzenden Habitaten nachgewiesen, sondern auch in Entfernungen von bis zu 30 km. Auf Basis dieser Daten konnten hochauflösende Vorhersagen des Pestizidvorkommens auf Landschaftsebene erstellt werden. Die Ergebnisse zeigen, dass komplexe Pestizidmischungen chronisch und ubiquitär auf landwirtschaftlichen Flächen, Nicht-Zielflächen und sogar in Naturschutzgebieten vorkommen. Auch in speziell für blütenbesuchende Insekten relevanten Kompartimenten wurden hohe Pestizidbelastungen festgestellt. Unterschiede zwischen Pollenproben von Wildbienen und Honigbienen weisen sowohl auf unterschiedliche Expositionen als auch auf eine hohe zeitliche Variabilität hin. Pestizidrückstände wurden zudem in frisch geschlüpften Wildbienen nachgewiesen. Basierend auf den gemessenen Bodenkonzentrationen wurden Risiken für Nicht-Zielorganismen berechnet. Insgesamt deuten die Ergebnisse dieser Dissertation darauf hin, dass die realistische Exposition von Insekten im aktuellen Zulassungsverfahren nicht ausreichend berücksichtigt wird und die Risiken der chronischen Exposition gegenüber Pestizidmischungen derzeit nicht adäquat bewertet werden.

1 Introduction

Global insect decline represents one of the most pressing ecological crises and poses a major scientific and societal challenge (Dicks et al. 2021). Substantial reduction in insect biomass, diversity, and abundance has been documented worldwide (Wagner et al. 2021; van Klink et al. 2024; van Klink et al. 2020; Outhwaite et al. 2022). Although synthetic pesticides are considered among the most strictly regulated chemicals (Brühl and Zaller 2019), they have been identified as a key driver contributing to insect decline (Dicks et al. 2021; Harvey et al. 2023) and biodiversity loss (Geiger et al. 2010). This is particularly concerning, as pesticides constitute the largest intentional input of biologically active substances into terrestrial ecosystems (Hvězdová et al. 2018).

During pesticide authorisation, toxicity studies, exposure modelling and risk characterisation procedures are conducted to ensure environmental protection. Nevertheless, increasing evidence indicates that chemical-synthetic current-use pesticides (CUPs) are widely detected far beyond their intended target areas. CUP residues have been recorded in environmental matrices, such as soils (Hvězdová et al. 2018; Silva et al. 2019; Froger et al. 2023), vegetation (Linhart et al. 2019; Linhart et al. 2021) and water bodies (Liess et al. 2021; Navarro et al. 2024). Various transport processes, including spray drift, runoff and atmospheric transport, can distribute CUPs (Reichenberger et al. 2007).

CUPs enter the atmosphere either during application as spray drift (primary drift) or after application through processes such as evaporation, vapour drift or wind erosion (secondary drift). Regulatory risk assessments estimate drift deposition using standardised field experiments, for example, using Petri dishes as collectors. However, these experimental setups may not fully represent environmental conditions, such as vegetation cover or heterogeneous surfaces. In addition to drift processes, the environmental fate of CUPs is further determined by dissipation processes, including degradation, volatilisation and redistribution among environmental matrices. Dissipation is commonly investigated under controlled conditions. However, field monitoring studies increasingly indicate that pesticide residues can persist in environmental matrices for extended periods (Riedo et al. 2023) and may accumulate due to repeated applications. Consequently, it remains unclear to what extent regulatory assumptions on drift and dissipation reflect CUP behaviour under realistic field conditions. To address this gap, a field study was conducted investigating drift and dissipation processes following a real-world CUP application (Paper 3).

Field studies further demonstrate that CUPs rarely occur as single substances; instead, complex CUP mixtures dominate environmental samples (Linhart et al. 2019; Silva et al. 2019; Brühl et al. 2021; Linhart et al. 2021; Brühl et al. 2024). However, the effects of CUP mixtures are rarely considered

beyond formulated products within the current regulatory framework. Experimental evidence indicates that combinations of CUPs can substantially increase toxicity. Notably, a recent study demonstrated that a mixture of three CUPs caused markedly higher toxicity than either the insecticide alone or a mixture containing only two active ingredients (Sedlmeier et al. 2025). Furthermore, the mixture of a fungicide and a herbicide has been shown to induce neurotoxic effects in insects (Di Noi et al. 2024). Further evidence that combinations of CUPs pose synergistic effects to non-target insects was provided by another study, which found that insects exposed to mixtures derived from environmental monitoring studies showed effects even when individual compounds were present at low concentrations (Gandara et al. 2024). Moreover, synergistic effects may occur when CUP exposure interacts with further stressors such as diseases, poor nutrition or additional CUPs (Bartling et al. 2024). Such interactions can lead to effects that exceed those from individual factors alone. Together, these processes highlight a potential discrepancy between regulatory risk assessment and real-world CUP exposure.

Insects living in agriculturally dominated landscapes face multiple exposure pathways through various environmental matrices across their life cycle (Figure 1). Exposure pathways include direct contact during CUP application or drift events, as well as indirect exposure due to contact with CUP-containing environmental matrices such as soil, vegetation, nectar, pollen or nesting material (Bartling et al. 2024). Contact with contaminated soil represents a particularly relevant exposure pathway, as many insects spend at least part of their life cycle in the soil (Anthony et al. 2023). Interactions with contaminated vegetation constitute other important pathways, as numerous species feed on plants, dwell on vegetation or use plants as shelter. Flowers play a central role for flower-visiting insects, as they provide nectar and pollen resources. Despite the ecological relevance of these matrices, these exposure pathways are only insufficiently considered in regulatory risk assessment (Uhl and Brühl 2019).

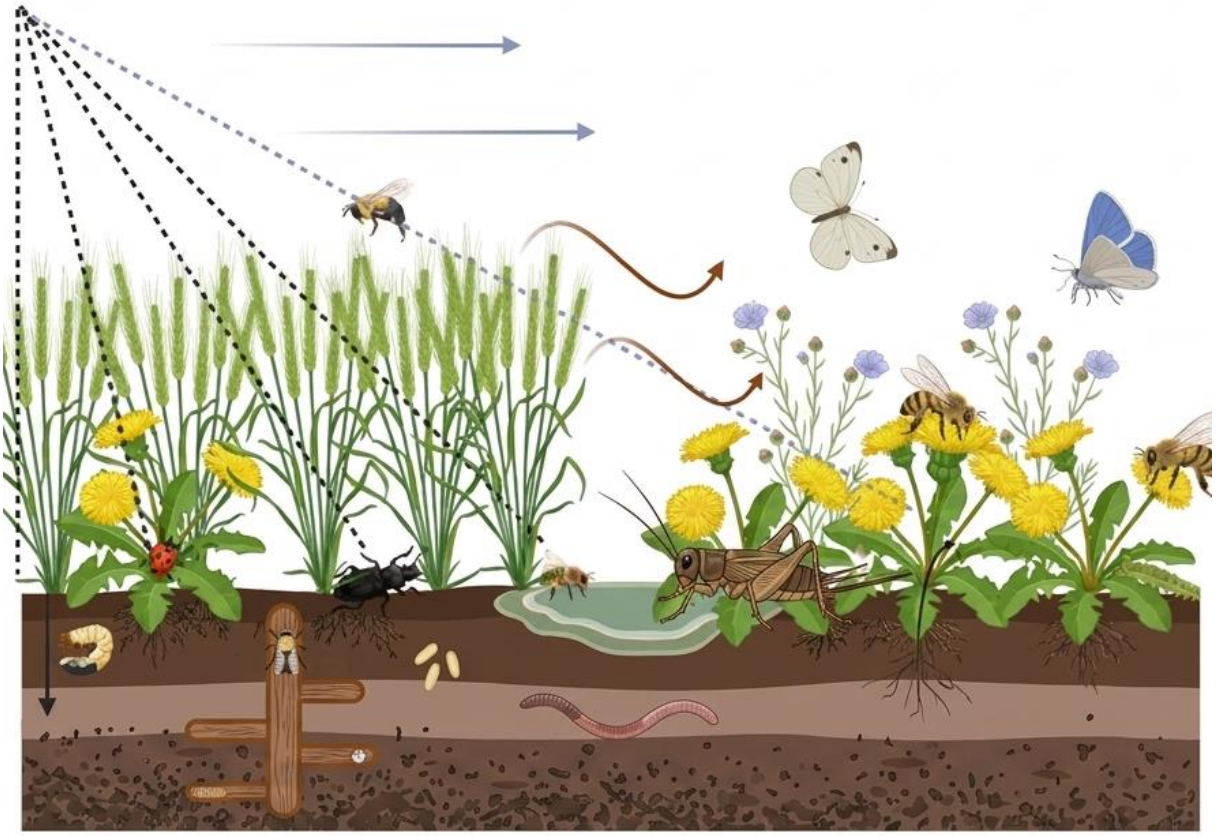


Figure 1: Potential exposure pathways of current-use pesticides to insects, in agricultural fields (left), as well as in adjacent non-target areas (right side), including direct spray (dashed black line), overspray (dashed blue line), primary spray drift (blue arrow), secondary drift (brown arrow). The graphic was prepared in biorender.com and optimised with Gemini 3.

Exposure to CUPs can lead to acute or chronic effects, ultimately leading to the death of the insect. However, the exposure can also lead to sublethal effects, which are characterised by physiological and behavioural changes (Alkassab and Kirchner 2017). Sublethal effects, such as reduced fertility, may lead to an overall reduction of offspring and therefore affect whole populations (Müller 2018; Albacete et al. 2024; Albaseer et al. 2025). Additional studies have demonstrated negative effects on navigation (Fischer et al. 2014) or learning behaviour (Kaila et al. 2023).

The recent revision of bee risk assessment guidance acknowledged regulatory gaps regarding exposure via soil or plant material. Yet, it stated that these pathways cannot currently be incorporated due to insufficient data (Adriaanse et al. 2023). Similarly, the consumption of contaminated water by insects is not addressed due to insufficient data (Adriaanse et al. 2023). Accordingly, the current regulatory risk assessment does not adequately address realistic exposure (Brühl and Zaller 2019).

In addition, regulatory pesticide risk assessments rely on a limited number of surrogates that are assumed to represent broader taxonomic groups. For pollinators, the Western honey bee (*Apis mellifera*) has historically served as the primary surrogate species for *Apis* and non-*Apis* bees (Adriaanse et al. 2023). Honey bees are used because they are easily available and can be maintained under laboratory conditions (Schmolke et al. 2021). However, honey bees differ substantially from

most wild bee species in their life history, behaviour and exposure pathways. Consequently, the extrapolation from one species to others without being routinely tested has been discussed as one reason for the ongoing insect decline (Linguadoca et al. 2022). In recognition of these limitations, recent regulatory guidance has expanded to include additional genera such as *Bombus* and *Osmia* (Adriaanse et al. 2023). Nevertheless, variability in CUP sensitivity has also been observed even within species (Schmolke et al. 2021; Linguadoca et al. 2022), suggesting that risk assessments based on a limited number of model organisms may not adequately capture the vulnerability of wild pollinator communities.

Despite these recognised limitations in exposure pathways and model species selection, empirical data on CUP residues in terrestrial environments remain scarce. Monitoring programmes covering large temporal and spatial scales are particularly required (Linhart et al. 2021; Cech et al. 2022; Sabzevari and Hofman 2022; Brühl et al. 2024), as agriculturally dominated landscapes constitute a substantial proportion of the terrestrial surface in Europe, including Germany, resulting in a mosaic of cultivated fields and adjacent non-target habitats such as field margins, grasslands and forests. Consequently, CUPs have the potential to affect a wide range of adjacent terrestrial environments. While monitoring programmes for CUP residues have been well established for aquatic environments for several decades, such as under the EU Water Framework Directive (Wolfram et al. 2021), comparable systematic monitoring approaches for terrestrial environments are still largely missing (Brühl et al. 2024). Recent European policy initiatives increasingly emphasise the importance of systematic soil monitoring for environmental protection (European Parliament 2026). The EU Soil Monitoring Law, developed under the EU Soil Strategy for 2023, aims to establish harmonised monitoring of soil health across the European Union and should also include the monitoring of pesticides (European Parliament 2026). To address the current knowledge gap of the occurrence and distribution of CUP residues in terrestrial matrices relevant to insects, this thesis included several complementary studies. These included a high-resolution year-round monitoring approach investigating seasonal CUP dynamics in soils and vegetation (Paper 4), a landscape-scale assessment of CUP distribution (Paper 5), and analysis of CUP residues in flowers, pollen provisions, and newly hatched adult bees (Papers 2, 6).

Quantifying pesticide exposure in terrestrial environments requires analytical tools capable of detecting a broad range of CUPs at trace concentrations across diverse matrices. Standard multiresidue methods (Anastassiades et al. 2003) require large sample amounts, which are not feasible in the context of environmental samples such as flowers, pollen-provision or single bees. Larger sample quantities are implementable for a composite sample, such as the pollen from different brood cells (Rusnáková et al. 2023) or in the context of bees, by the combination of several individuals (Böhme et al. 2018). While this also gives an overview of CUP presence in the environment, mixed samples lose

information about the individual situation and risk (David et al. 2015). However, understanding insect exposure to CUPs depends on precisely such small-scale matrix-adapted analytical approaches. To overcome these analytical limitations, matrix-adapted multiresidue methods were developed for soils and vegetation (Paper 1) and for flowers, pollen provisions, and individual wild bees, enabling the direct quantification of CUP residues in matrices especially relevant for flower-visiting insects and in the insects themselves (Paper 2). These analytical developments provide the methodological foundation for the environmental monitoring studies presented in this thesis.

Despite increasing evidence that CUPs are widely distributed in terrestrial environments, substantial knowledge gaps remain regarding their occurrence, persistence, and distribution across terrestrial environmental matrices relevant to insects. In particular, field data on the environmental fate of CUPs, empirical data on CUP mixtures, realistic exposure pathways, and the spatial and temporal dynamics of CUPs in agricultural landscapes are still limited. This thesis, therefore, aims to investigate the spatial and temporal distribution of CUP residues across terrestrial matrices relevant to insects, including soils, vegetation, flowers, pollen provision, and insects themselves. By applying complementary matrix-adapted multiresidue methods and analysing more than 1,000 environmental samples, this work provides a high-resolution assessment of CUP mixtures across terrestrial matrices and seasons. The resulting data allow an integrative evaluation of CUP exposure in agriculturally dominated landscapes and provide a basis for discussing the implications for environmental risk assessment and the protection of non-target insects.

2 Objectives and Thesis Structure

This thesis aimed to characterise the CUP distribution in agriculturally dominated landscapes to understand insect exposure to CUPs (Figure 2). For this purpose, method development was combined with complementary field studies.

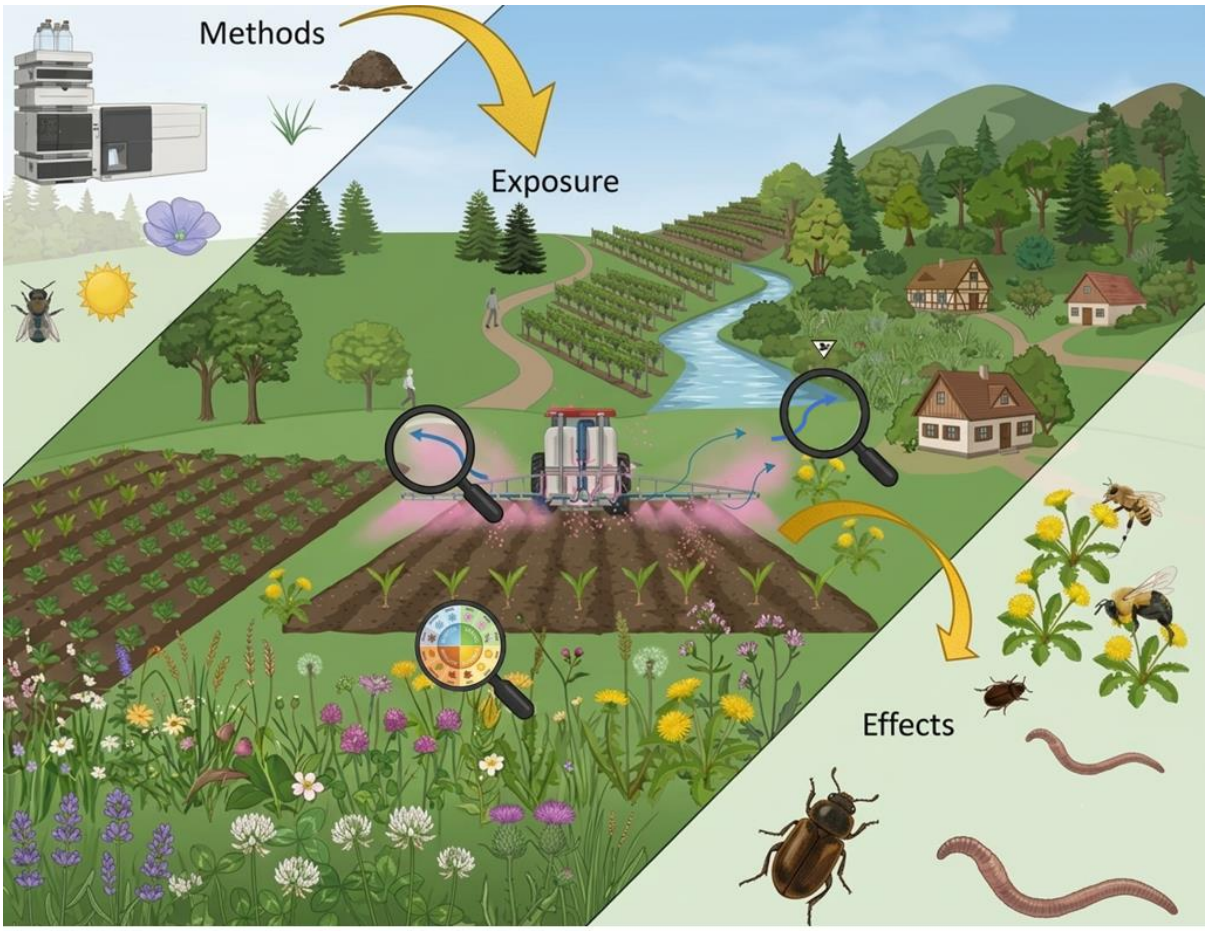


Figure 2: Conceptual overview of the three objectives addressed in this thesis. The graphic was prepared in biorender.com and optimised with Gemini 3.

The first objective of this thesis was to develop robust, simple and cost-efficient extraction methods for the identification and quantification of synthetic current-use pesticide (CUP) residues in complex environmental matrices, particularly in insect-relevant matrices. Extraction methods were established for soil and vegetation (Paper 1), as well as for flowers, pollen and wild bees (Paper 2). Together, these methods form the analytical basis for all subsequent field-based investigations and enable the assessment of multiple exposure pathways relevant to insects in agricultural landscapes.

The second objective aimed to characterise the occurrence and distribution of CUPs in terrestrial environments relevant to insect exposure. A focus was set on the occurrence and persistence of CUPs

across different spatial and temporal scales under field-realistic conditions. Drift and dissipation processes were investigated following CUP applications under real-world conditions, but under agricultural best practice and favourable conditions, with 90%-drift-reducing technology and without wind, at small spatial scales ranging from the field to adjacent non-target areas (up to 20 m off-field, Paper 3). The temporal dynamics of CUP residues were assessed by monthly sampling over a period of one year at the same spatial scale with representative agricultural use types (Paper 4). This approach allowed the evaluation of potential persistence and accumulation of CUPs in both treated and adjacent non-target areas. The spatial extent of CUP distribution was examined at the landscape scale by analysing residues in non-target areas several kilometres away from treated fields and by model predictions (Paper 5). Finally, to capture exposure pathways particularly relevant to flower-visiting insects, CUP residues were further analysed in flowers, pollen provisions, and wild bees (Paper 2), as well as in honeybee-collected pollen (Paper 6).

The third objective was to evaluate potential effects and risks arising from the detected CUP exposure for non-target organisms, with a particular focus on insects. This objective builds on the exposure patterns identified under the second objective and integrates residue data with ecotoxicological endpoints to assess potential implications for earthworms, Collembola and insect populations (Papers 2, 3, 6).

3 Methods Overview

3.1 Method Development

Extraction methods enabling the simultaneous detection of multiple CUPs in small environmental and biological samples were developed and validated as an analytical basis for the monitoring approaches applied in this thesis. The methodological approach follows the description in Papers 1 and 2.

Blank material was collected or obtained for method development, including topsoil and vegetation collected near Jockgrim, Germany (49°05’10.3’’N 8°15’33.1’’E) and flowers in Landau, Germany (49°12’13.7”N 8°06’20.2”E), where, within a radius of 1 km, no agricultural fields were present. Commercially available pollen (Blütenpollen Alpenmischung, GRAZE der Imkershop, Weinstadt, Germany) and adult red mason bees (*Osmia bicornis*, WAB-Mauerbienenzucht, Konstanz, Germany) were purchased. The bees were obtained as cocoons and hatched in the laboratory for method development.

To evaluate extraction efficiency (recovery), blank material was spiked with a CUP-mixture containing 98 CUPs (pre-spike). The results were compared to blank samples spiked after the extraction (post-spike) to determine recovery rates. Extraction methods were based on acetonitrile acidified with formic acid as the solvent; samples were agitated, centrifuged and filtered before analysis. Quantification of CUP residues was performed using high-performance liquid chromatography coupled with tandem mass-spectrometry by electrospray ionisation (HPLC-ESI-MS/MS).

As agitation and extraction time are important steps, different methods were tested for soil samples, including hand-shaking, sonication and end-to-end-shaking. To minimise matrix effects and interferences in pigment- and fatty-acid-containing matrices, a clean-up step was necessary. Different approaches were tested, including the use of GCB, PSA, C18, Z-Sep and a freeze-out step. For wild bees, an ultrasonic bath extraction method established for small-sized aquatic insects was tested (Roodt et al. 2023) and compared to the acetonitrile-based extraction adapted from the extraction of soil and vegetation, followed by different clean-up approaches. Due to differences in weight between female and male bees, extraction methods for wild bees were adapted to sex-specific sample masses. Methods with the best recovery results were selected and validated against common criteria (SANTE, EC), including recovery, relative standard deviation, linearity, matrix effect, and quantification and detection limits.

3.2 Study Sites

Samples for the monitoring studies were collected in agriculturally dominated landscapes in southwestern Germany (Figure 3). Most field investigations were conducted in the Palatinate region and Baden, located in the Upper Rhine Valley, while honey bee pollen samples were investigated in Lower Franconia, Bavaria. The Upper Rhine Valley is a large valley bordered by the Palatinate Forest and the Odenwald to the north, and the Vosges Mountains and the Black Forest to the south. The region is characterised by a high environmental heterogeneity and is considered a biodiversity hotspot in Central Europe. (Ackermann et al. 2012). At the same time, the area is used for intensive agricultural production (Schwieder et al. 2020) and contains a mosaic of agricultural and protected areas, such as Natura 2000 sites, nature conservation areas and biosphere reserves (Federal Agency for Nature Conservation 2025). The Palatinate region is part of the Upper Rhine Valley and is characterised by highly diverse agricultural land use. Viticulture represents one of the dominant agricultural cultivations, with more than 20,000 hectares of vineyards in the Palatinate wine-growing region (Abele 2024). In addition, large areas are used for vegetable production, orchards and arable crops. In particular, vegetables are cultivated in northern Palatinate, while arable farming dominates the southern part of the region (Schwieder et al. 2020). The sampling sites in Lower Franconia were located along a landscape gradient ranging from 43% to 93% of the surrounding landscape covered by annual cropland. This region is characterised by a heterogeneous landscape, including croplands, forests, grasslands, and urban areas. Although Lower Franconia is also a wine-growing region, viticulture was not present at the specific sampling sites. The characterisation of both regions makes them suitable case study regions for investigating the distribution of CUPs in environmental matrices relevant to insect exposure.

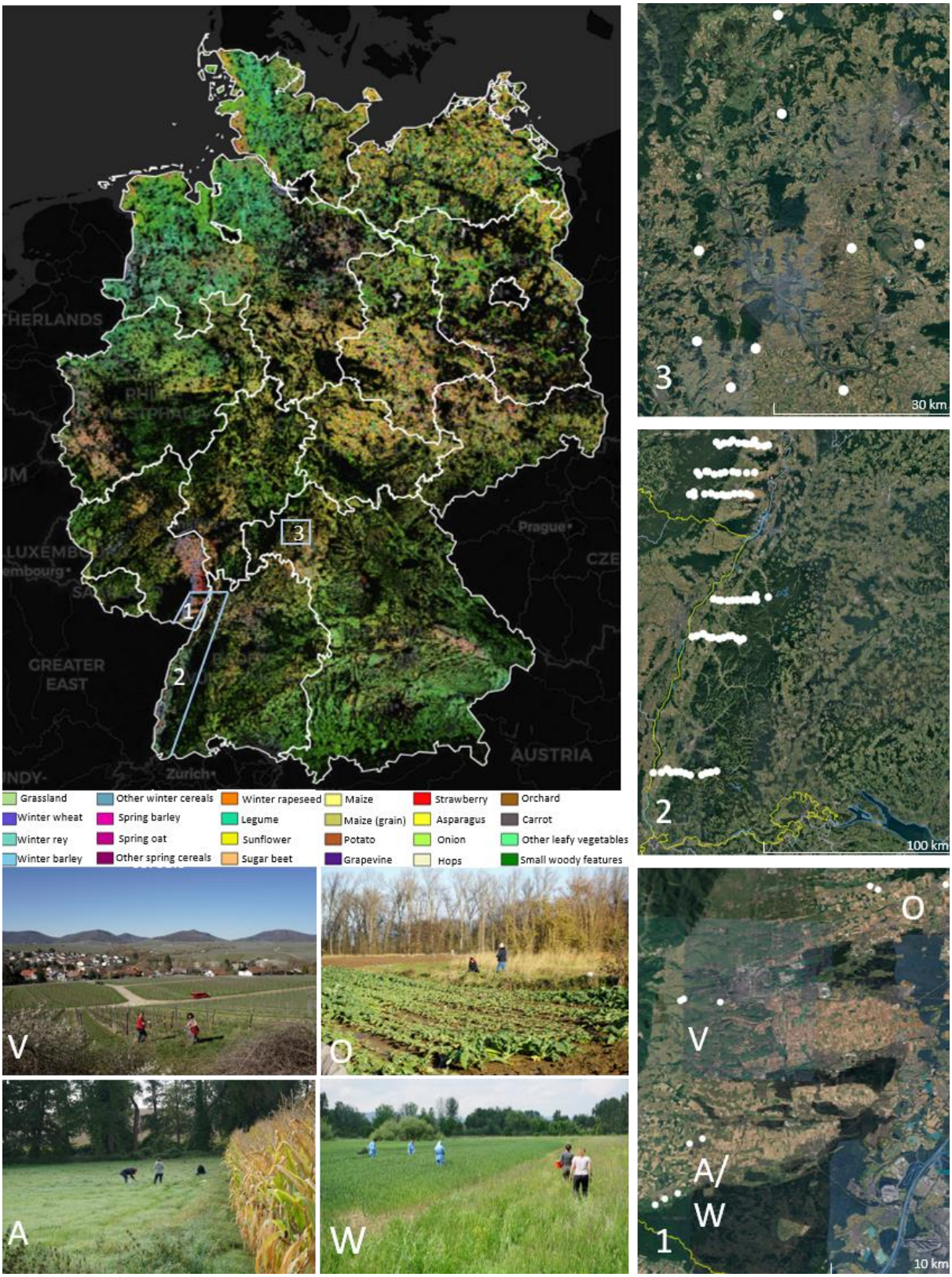


Figure 3 Area of the study sites in the context of German agricultural land-use (upper left picture (Blickensdörfer et al. 2022)) and aerial view (Google Earth®) on the sampling areas (3: honey bee pollen study, 2: landscape-scale study, 1: year-round and environmental fate study) with the approximate locations of the sampling sites (white dots). Photos of the sampling sites from the year-round study (V: viticulture, O: vegetable crops, A: arable crops) and from the environmental fate study (W: winter cereals).

3.3 Environmental Fate Study

The environmental fate study corresponds to the experiment design described in Paper 3 (Figure 4). In March 2023, a pesticide application on three winter wheat fields in the southern Upper Rhine Valley in Germany was investigated under real-world, but best-case conditions, including low wind, optimal temperatures and drift-reducing techniques. The fields were situated in proximity, with each field having an adjacent meadow aligned with the main wind direction. Four CUPs, one pirimicarb-based insecticide and a fungicide mixture containing bixafen, fluopyram, and prothioconazole were applied by the farmer. Applications were conducted sequentially on the three fields under best-case weather conditions using 90% drift-reducing nozzles. A sample of the tank mixture was taken between the applications. To quantify primary spray drift, Petri dishes were placed within the fields and in adjacent meadows at distances of 1, 5 and 20 m from the field, before the application (Figure 4). Petri dishes were collected immediately after spraying. Topsoil and vegetation samples were taken at least one hour after application. Topsoil (0–5 cm) was sampled separately as bare soil and vegetation-covered soil. Vegetation was collected from standardised volumes (20 × 20 × 20 cm). Each sample consisted of three subsamples taken 5 m apart along the same distance. Samples were transported under cooled conditions and stored at −20 °C until analysis. All environmental samples were analysed using the validated multiresidue methods developed in this thesis for soil and vegetation (Paper 1). Petri dishes were extracted with acetonitrile, and the tank mixture sample was diluted before direct injection. In addition to the validated CUPs, prothioconazole and its metabolite, prothioconazole-desthio, were analysed.

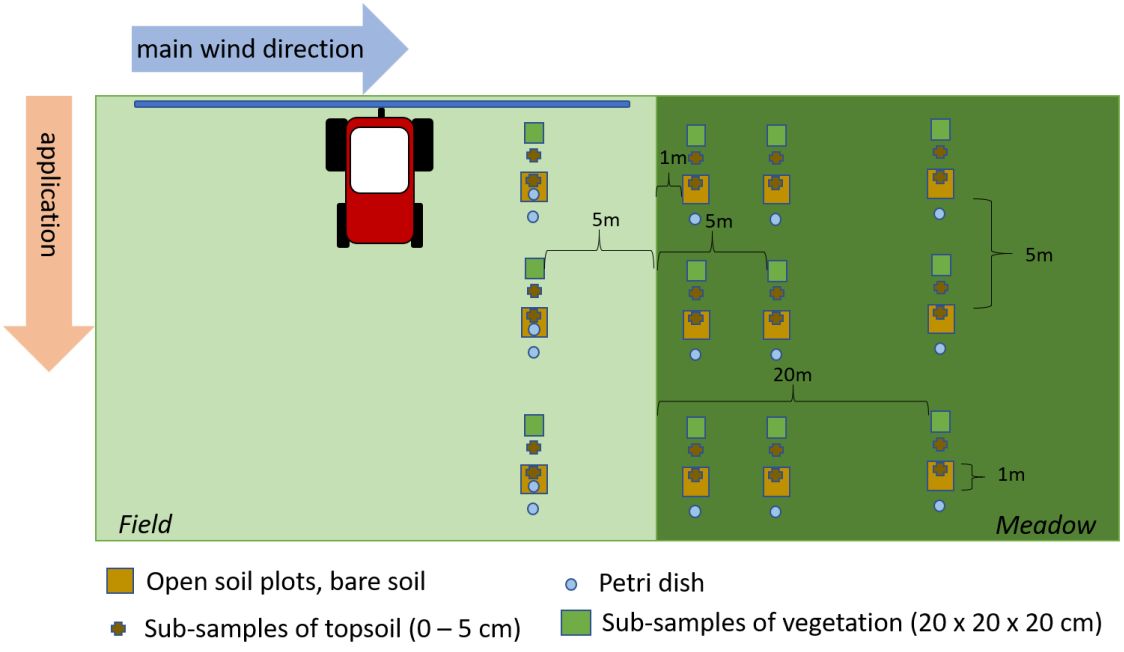


Figure 4 Sampling design of winter wheat fields and adjacent meadows at the three study sites. At each site, bare and covered topsoil, vegetation and Petri dishes were sampled right after the application of four CUPs in the field and 1, 5 and 20 from the field edge into the meadow. Each sample consisted of three sub-samples spaced 5 m apart. Figure taken from Paper 3.

To assess dissipation, topsoil and vegetation were sampled repeatedly up to 128 days after application. Bare topsoil was sampled for only 8 days after application due to subsequent vegetation cover. Vegetation sampling continued for 64 days, limited by harvesting and mowing events. Dissipation was also assessed by a dissipation model, MIxture Toxicity of Application Spray series (MITAS), which was initially developed to estimate mixture toxicity (Sybertz et al. 2020).

3.4 Temporal and Spatial CUP Distribution

The year-round monitoring study follows the methodological framework described in Paper 4 (Figure 5). From February 2021 to February 2022, monthly topsoil and vegetation samples were collected from nine agricultural fields and their adjacent meadows in the Palatinate region (Germany). To reflect the heterogeneity of agricultural land use in this region, three dominant production systems were included: arable crops, vegetable growing and viticulture. Each system type was replicated in three sites. The arable sites were located in the southern Palatinate near the French border, vegetable production sites were situated close to the Rhine River, and viticulture sites were distributed along the Southern Wine Route. These land-use types differ substantially in management intensity, crop rotation and application techniques. Viticulture is a permanent crops and three-dimensional spraying systems, whereas arable crops and vegetables are managed in two-dimensional spraying systems with annual or short crop rotations, respectively. At each site, samples were collected from the cultivated field and from the adjacent meadow located downwind (west to east) to capture potential worst-case drift scenarios (Figure 5). Samples in the adjacent non-target meadows were taken at distances of 1, 5 and 20 m from the field. Topsoil sampling (0–5 cm) following the protocol described in Section 3.3. A standardised volume of vegetation was hand-collected.

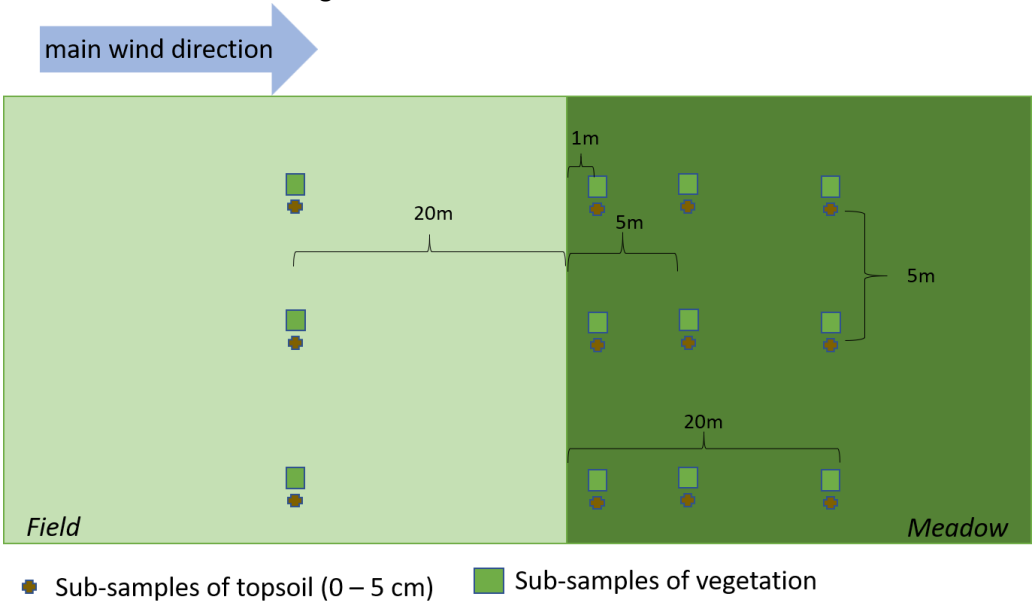


Figure 5 Sampling design of agricultural fields and adjacent meadows at the nine study sites for the year-long study. At each site, topsoil and vegetation were sampled monthly in the field and 1, 5 and 20 from the field edge into the meadow. Each sample consisted of three sub-samples spaced 5 m apart. Figure adapted from Paper 4.

3.5 CUP Characterisation at the Landscape Level

To extend the monitoring approaches to a broader scale, a landscape-scale study was conducted, and field data were used to predict CUPs at high resolution. This methodological approach follows the description in Paper 5. Between mid-June and mid-July 2022, topsoil, vegetation and water were sampled along six transects with an orientation following the main-wind direction (west to east) and 30 km length in the Upper Rhine Valley (Figure 6). Each transect consisted of 13 sites, with six sites placed in the valley, one close to the base of the mountain and six on the mountain slope. Topsoil (0-5 cm) was sampled in 25 sub-samples taken from an area of 4 x 4 m per sampling point. Vegetation was handpicked in the same areas. Water samples were taken only if water resources, such as lakes or puddles, were available. Topsoil and vegetation were analysed according to the described method (Paper 1), and water was analysed using a direct-injection approach for 74 CUPs (Roodt et al. 2023).

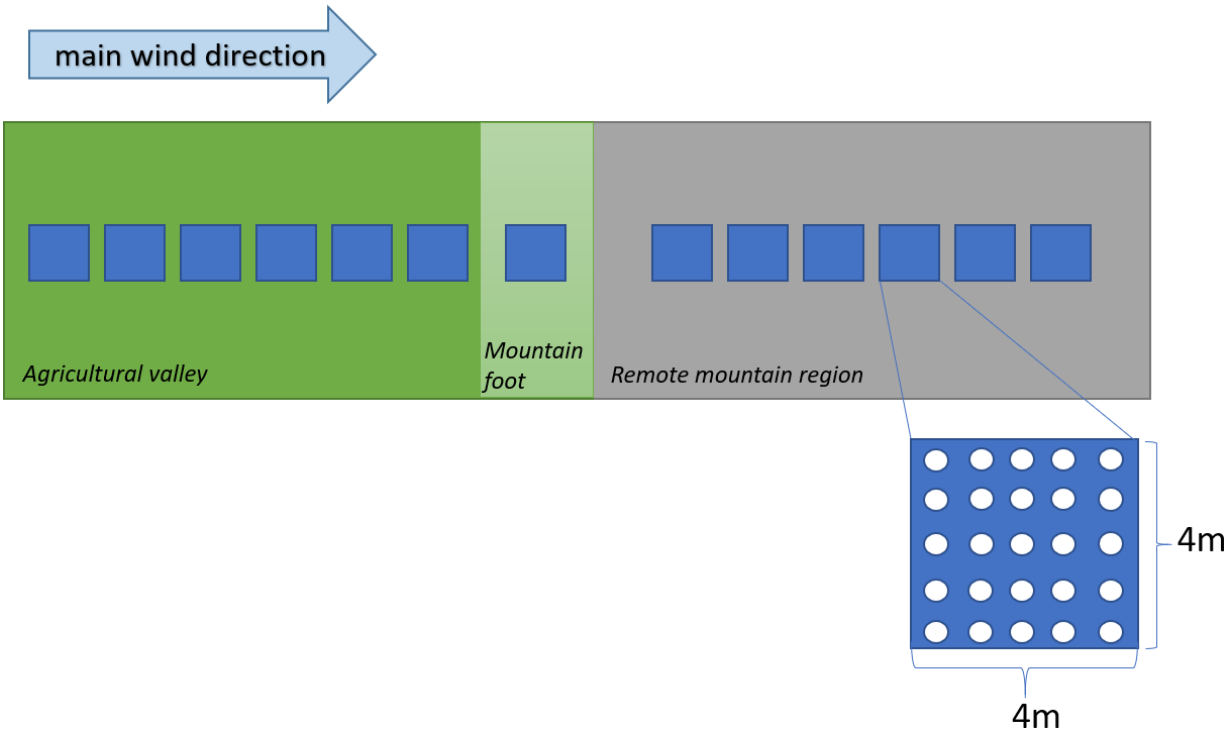


Figure 6 Sampling design of the landscape-scale study. Along 30 km of slope transects at 13 sites, topsoil and vegetation samples were collected: six in agricultural valleys, one at the base of the mountain, and six on the mountain slope. Per sampling point, a sub-sample (white dots) of 25 topsoil or vegetation samples was collected from a 4 x 4 m area (blue square).

To predict CUPs across an 11,000 km^2 area in the Upper Rhine Valley, land-use weights were identified by analysing the surrounding land-use composition and applying a two-dimensional Gaussian kernel to land-use raster cells based on their distances from the sampling sites. The resulting land-use weights predicted CUP numbers and concentrations in topsoil and vegetation at a landscape level with high resolution.

3.6 Flowers, Pollen Provision and Bees

To analyse CUP exposure, especially relevant for flower-visiting insects (FVI), flowers, pollen provision from *Osmia bicornis* and *Apis mellifera*, as well as newly hatched *O. bicornis,* were investigated following the methodology described in Papers 2 and 6. Representative flowers were handpicked along the meadows from the year-round monitoring study (Section 3.4, Paper 4) from March to June 2021. The flowers were analysed using the validated multiresidue method for flowers (Paper 2).

Osmia species are solitary wild bees with a ventral surface of the abdomen for pollen carrying. *O. bicornis* (Figure 7B), or red mason bee, is a common species in Germany, living in diverse habitats, using a broad range of flowers as a resource (Westrich 2026). They build their nests in holes or tubes with linearly arranged, partitioned cylindrical cells, separated by mortar (Figure 7A). *O. bicornis* is a robust species in comparison to other wild bees and is therefore also suitable for investigations. Vulnerability and ability to recover is highly diverse among bee species, with non-*Apis* bees being potentially more vulnerable than honey bees (Schmolke et al. 2021). However, variability has been observed not only between species but also within species, as demonstrated for *O. bicornis* (Linguadoca et al. 2022). As described in Paper 2, *O. bicornis* was used to investigate insect exposure to CUPs in pollen provision and in individual bees. *O. bicornis* cocoons were placed continuously under wooden nesting aids at the sample sites from the year-round study in the Upper Rhine Valley from the end of March to the end of July 2021. The pollen provision collected by the female bees was sampled and analysed according to the validated multimethod for pollen (Paper 2). Nesting aids were transferred in cooler conditions for the overwintering period, after which cocoons were taken off, and bees were allowed to hatch. The hatched bees were immediately frozen and later analysed using the validated multiresidue method (Paper 2).

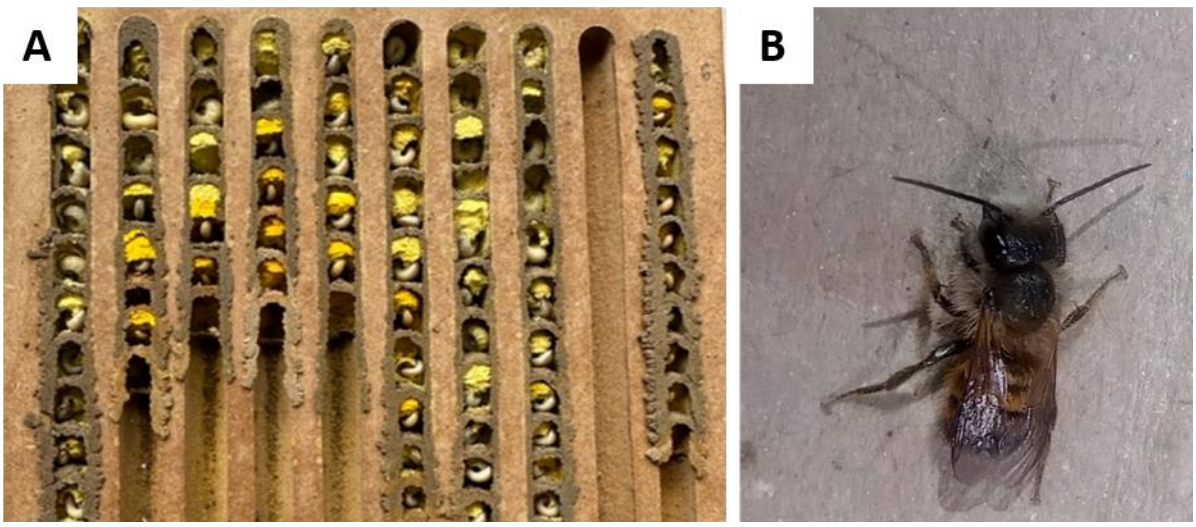


Figure 7 A) Brood cells of Osmia bicornis in nesting aids placed at the sampling sites of the year-round study next to an agricultural field. Pollen provision from certain brood cells was measured for CUP residues; other pollen provision was left in the cells, and adult bees were measured after their hatching. B) A O. bicornis adult after hatching.

Like *O. bicornis*, *Apis mellifera* is a polylectic generalist using many different plants as a flower and pollen supply (Layek et al. 2020; Lowe et al. 2023). *A. mellifer*a collects pollen provision within a large flight radius of approximately 2 km (Danner et al. 2016). In contrast to *O. bicornis*, *A. mellifera* collects pollen with pollen combs on its legs. They live in social structures and overwinter in their nests, while adult *O. bicornis* live for only one season. To compare CUP exposure for different FVI, *A. mellifera* colonies were established at the centre of nine sampling sites in Lower Franconia from mid-June to mid-August 2021. For colony establishment, sampling was started after three weeks. After these three weeks, pollen traps were placed in front of the hives for one day every three days from July to August (Figure 8). Pollen from four hives was used for DNA metabarcoding and from one hive for CUP analysis following the validated multiresidue method for pollen (Paper 2).

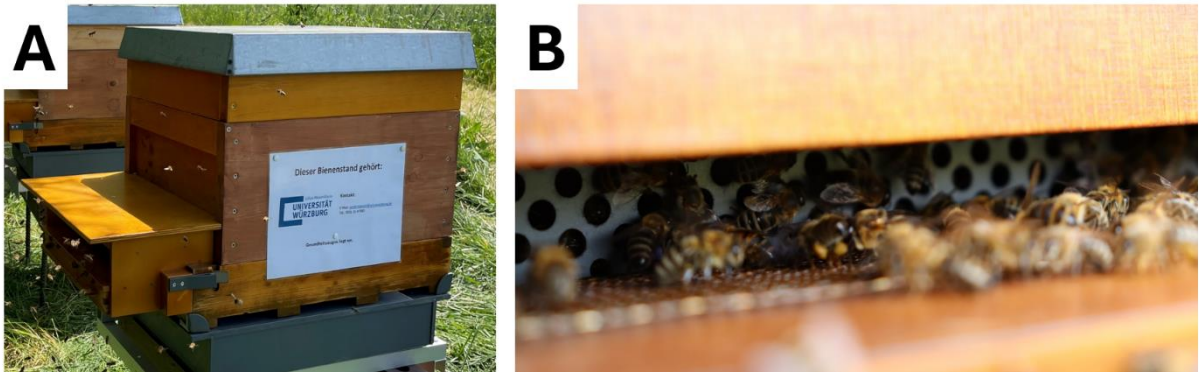


Figure 8 Figure according to Paper 6: A) Pollen trap manufactured by the workshop of the Julius-Maximilians-Universität Würzburg with a protruding roof as weather protection and a drawer for pollen collection. B) Honey bees passed the grid before the entrance and brushed off pollen panties, which fell through another grid at the bottom (unpassable by honey bees) into a drawer.

3.7 Mixture Risk Calculations

In contrast to standard single-substance assessments, an exposure-based mixture risk calculation was conducted using measured environmental concentrations from the year-long monitoring study (Paper 4). Due to conceptual limitations in current regulatory frameworks, risk quotients were calculated for earthworms, springtails and ground-nesting wild bees using publicly available regulatory toxicity endpoints. For earthworms, a published approach for risk calculations based on measured concentrations (Vašíčková et al. 2019; Pelosi et al. 2021; Panico et al. 2022; Froger et al. 2023) was used and adapted for collembola. As species-specific toxicity data for ground-nesting wild bees are currently lacking, honey bee endpoints were used as a surrogate following a published approach (Willis Chan et al. 2019). Only concentration addition was considered for mixture assessment; potential synergistic or antagonistic interactions could not be incorporated; therefore, a conservative default approach in mixture toxicology was performed.

General Discussion

4 Development of Extraction Methods (Objective 1)

The following sections describe the development of analytical methods for detecting CUPs in terrestrial environmental matrices relevant to insects. The methods were designed to enable the analysis of small sample quantities from diverse matrices and to serve as the methodological basis for the environmental monitoring studies presented in this thesis (Papers 1–2).

4.1 Challenges of Extracting and Quantifying CUPs From Different Matrices

Characterising insect exposure to CUPs under realistic environmental conditions requires analytical methods capable of simultaneously detecting a broad range of compounds in small environmental samples. Environmental matrices relevant to insects differ substantially in their properties, posing substantial analytical challenges. For example, soils represent complex matrices because they contain components such as soil organic matter that can interact or bind with CUPs (González-Curbelo et al. 2022) and thereby complicate residue extraction. The matrix itself can change the analytical signal and lead to matrix effects, resulting in signal enhancement or suppression (Rösch et al. 2023). Matrix-matched calibration can reduce these effects (Kwon et al. 2012; Giacinti et al. 2016), yet extraction procedures should be optimised to minimise matrix interferences. While numerous extraction methods exist for CUP analysis in soils, limited studies have addressed CUP extraction from vegetation. Nevertheless, CUP residue analyses originate from food matrices, and several approaches developed for leafy vegetables can be adapted for vegetation. Plant matrices contain pigments, lipids and other compounds that may be co-extracted when organic solvents are applied. Therefore, additional clean-up steps are necessary to remove co-extracted matrix components and protect the analytical instrumentation. The development of a simplified extraction protocol applicable across several environmental matrices therefore requires balancing efficient CUP extraction with the removal of interfering compounds (Figure 9).

Matrix-specific clean-up strategies were applied to minimise matrix effects. In vegetation samples, a clean-up step using graphitised carbon black (GCB) was necessary to remove chlorophyll and reduce matrix effects. However, GCB was not suitable for matrices associated with FVI. For flowers, pollen provisions and insect samples, an overnight freeze-out step was therefore applied to remove lipids and fatty acids (Figure 9). This approach proved efficient and robust across all FVI-related matrices and was therefore adopted as the standard clean-up procedure for these samples.

All methods were based on an acetonitrile extraction procedure, which was used in the fundamental extraction approach for multiresidue pesticide analysis (Anastassiades et al. 2003). Acetonitrile is an efficient solvent for extracting a broad range of pesticides, while having a low rate of co-extracting matrix components such as lipids or pigments compared to solvents like ethyl acetate (Anastassiades et al. 2003). In the developed methods, ammonium formate was used as an alternative buffer salt to the original QuEChERS salt, as it is more volatile than other salts and therefore theoretically results in less maintenance for the mass spectrometer (González-Curbelo et al. 2014). The addition of formic acid to acidify the extract likely contributes to enhancing extract stability (Acosta-Dacal et al. 2021) and may have improved the recovery of both acidic and basic CUPs. This suggests that slight adjustments in solvent chemistry can influence analyte preservation and extraction efficiency, particularly when compounds with differing ionisation properties are targeted simultaneously. Despite the broad applicability of multiresidue methods, certain pesticides cannot easily be included due to their specific physicochemical properties. For example, highly polar compounds such as glyphosate require dedicated analytical approaches and are therefore difficult to analyse (Fernández-García et al. 2025) and to integrate into large multiresidue screening methods.

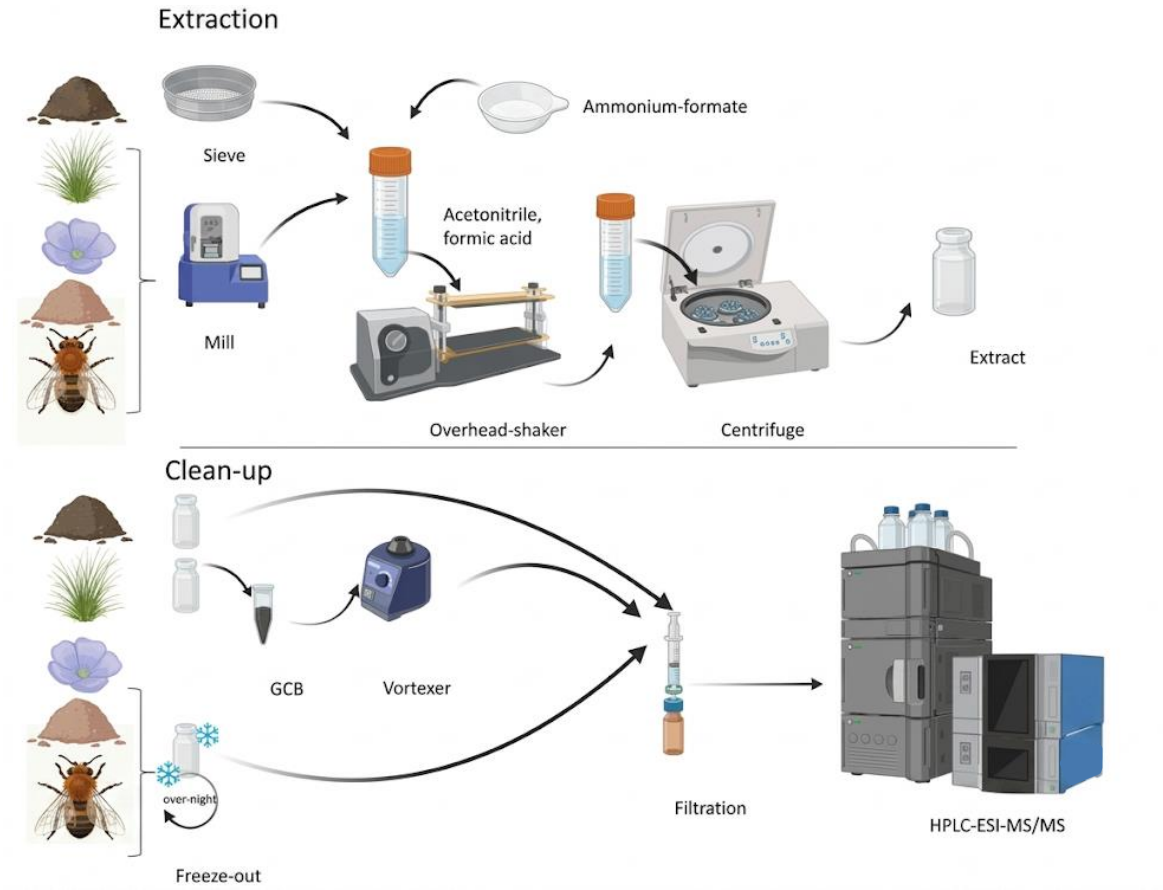


Figure 9 Diagram of the developed extraction procedures for multiresidue analyses of CUP from environmental matrices (soil, vegetation, flowers, pollen and bees) and the following clean-up steps, which involve graphitised carbon black (GCB) for vegetation and freeze-out overnight for flowers, pollen and bees. After a filtration step, samples were analysed using high-performance liquid chromatography coupled with tandem mass-spectrometry by electrospray ionisation (HPLC-ESI-MS/MS). The graphic was prepared with biorender.com.

4.2 Implication for Monitoring Approaches

The developed methods enabled the simultaneous analysis of multiple CUPs using comparatively small sample amounts. Suitable sample amounts were identified as 5 g dry weight (d.w.) of soil and 1 g d.w. of vegetation (Paper 1) and 0.5 g d.w. of flowers, 0.1 g fresh weight (f.w.) of pollen provision and 0.02 and 0.01 g d.w. of bees (Paper 2, Table 1). These small sample amounts minimise ecological disturbance during sampling and correspond to environmentally relevant biological units, such as the pollen provision for a single *O. bicornis* larva and the mass of an individual *O. bicornis* bee. Analysing individual bees rather than pooled samples preserves information on individual exposure while reducing environmental impact.

The methods developed in this thesis were validated to achieve comparably low LOQs despite reduced sample volumes, thereby enabling the detection of environmentally realistic background concentrations. This is particularly important in monitoring contexts, where low and chronic exposure levels (Chiaia-Hernandez et al. 2017) rather than acute peak concentrations are expected. Moreover, reduced analytical sensitivity results in fewer detections and may therefore underestimate environmental exposure (Solla et al. 2012). At the same time, a small sample volume can negatively impact sensitivity (SANTE 11312/2021). Achieving an appropriate balance between ecological relevance and analytical performance, therefore, represents a methodological challenge.

Soil and vegetation represent matrices that are comparatively easy to sample and are therefore well-suited for large-scale monitoring approaches with minimal disturbance of the ecosystem. As these compartments constitute key habitats for many insects (Uhl and Brühl 2019), the extraction method prioritised a broad CUP coverage and comparatively larger sample size. In contrast, matrices directly associated with FVI are often only available in very small quantities and are seasonally restricted. In line with this, the number of target CUPs decreased and achievable LOQs increased from environmental to biological matrices (Table 1). Nevertheless, the achieved sensitivities are within the range reported for comparable analytical approaches and allow a consistent assessment of potential insect exposure across environmental matrices.

Table 1 *Overview of the extraction methods: sample amount, number of CUPs, limit of quantification (LOQ), linearity (R^2), relative standard deviation (RSD), recovery (REC) and the clean-up procedure for each matrix (soil and vegetation (Paper 1) and flowers, pollen provision and female and male O. bicornis (Paper 2). Validation was conducted according to published criteria (SANTE 11312/2021; SANTE 11813/2017).*

Matrix	**Sample amount (g)**	**CUPs**	**LOQ (µg kg^{-1})**	**R^2**	**RSD (%)**	**REC (%)**	**Clean-up**
Soil	5 (d.w.)	98	0.008 – 21.5	> 0.99	1 – 14	71 – 125	-
Vegetation	1 (d.w.)	98	0.008 – 21.5	> 0.99	2 – 13	70 – 117	GCB
Flowers	0.5 (d.w.)	83	0.25 – 50	> 0.99	3 - 15	72 – 118	Freeze-out
Pollen provision	0.1 (f.w.)	71	0.2 – 52	> 0.99	4 - 13	74 – 116	Freeze-out
O. bicornis, female	0.02 (d.w.)	65	0.2 - 80	> 0.99	5 – 14	76 - 114	Freeze-out
O. bicornis, male	0.01 (d.w.)	45	0.08 - 100	> 0.99	6 – 15	78 – 112	Freeze-out

From a monitoring perspective, matrices associated with FVI, such as flowers, pollen or insects themselves, are more difficult to obtain, due to their seasonal restrictions and as their sampling involves stronger ecological disturbance in comparison to soil and vegetation. The latter represent particularly suitable matrices, as they are widely available, comparatively easy to sample and closely linked to insect habitats. Analysing these matrices allows for repeated measurements with limited disturbance of the ecosystem and therefore provides a robust basis for long-term monitoring approaches. Stratified monitoring approaches with high-resolution monitoring of CUP residues in topsoils and vegetation in representative regions with broader surveillance networks could therefore represent a feasible strategy to detect systemic CUP patterns and mixture complexity in terrestrial environments. Such monitoring could help to validate model-based exposure assumptions conducted in authorisation processes and identify spatial CUP patterns that remain undetected in standard regulatory scenarios. Establishing harmonised soil monitoring programmes across Europe, including pesticide monitoring, as currently planned under the EU Soil Monitoring Law (Directive - EU - 2025/2360 - EN - EUR-Lex 2026). It highlights the increasing political recognition of soil as a critical environmental matrix. However, systematic monitoring of pesticide residues in other terrestrial matrices, such as vegetation, is still not discussed, despite their potential relevance for biodiversity and ecosystem functioning.

Currently, approximately 300 active ingredients, including chemical-synthetic and natural or nature-identical active substances, are authorised for use in Germany (BVL 2025). The multiresidue methods developed in this thesis cover up to 98 CUPs. Although this represents only about one third of the authorised CUPs, most multiresidue approaches cover between 30 and 80 CUPs, while only a few methods include more than 100 analytes (Rösch et al. 2023). Pesticides that are no longer authorised may still be detected in environmental samples due to their persistence, as demonstrated for legacy compounds such as DDT (Silva et al. 2025) and may be useful to integrate into monitoring approaches. However, continuous adaptation of analytical target lists is complex, time-consuming and comes along with costs. The subset selected for the presented methods was based on the most frequently used CUPs in Germany (PAPA - Julius Kühn Institut 2019) and on compounds suitable for multiresidue analysis. While the methods developed here prioritised frequently used CUPs, other monitoring strategies set further focus on compounds with long environmental persistence (Rösch et al. 2023) or on CUPs previously detected in environmental samples (Giusti et al. 2018). The composition of the analytical target list inevitably shapes the resulting residue patterns.

Due to their frequent application, fungicides are more frequently represented among the targeted compounds than herbicides or insecticides. This imbalance may influence the observed distribution of pesticide groups and could result in an underrepresentation of compounds with fewer targeted analytes, particularly insecticides. Therefore, the findings should always be interpreted in the context of the selected target list. In addition, results are likely to underrepresent the total CUP load. Nonetheless, in order to identify important CUPs for terrestrial monitoring, initial monitoring based on usage data is advisable. Using the developed methods, CUP characterisation was assessed across temporal and spatial scales in agriculturally dominated landscapes to better characterise realistic exposure of insects to CUPs.

5 Characterisation of CUPs in Terrestrial Environments (Objective 2)

The following sections examine the occurrence and distribution of CUPs in terrestrial environmental matrices relevant to insects in agricultural landscapes. These matrices may represent important exposure pathways and are investigated based on the studies presented in Papers 2–6.

5.1 Primary Pathways of CUP Entry into Non-Target Terrestrial Areas

The unintentional contamination of terrestrial non-target areas is often caused by CUP drift during and after the application in fields (Albaseer et al. 2025). Primary drift occurs during application, where all mixed CUPs are theoretically present in each spray droplet. This primary drift is influenced by the application technique (Carlsen et al. 2006), dynamic surface tension (Schampheleire et al. 2009) and formulation rather than the active ingredients. In contrast, the drift investigations conducted in this thesis showed different spray drift for four simultaneously applied CUPs on Petri dishes (Table 2). This indicated that the drift of different CUPs within a tank mixture occurs individually and that not all CUPs end up with the same extent of primary drift (Paper 3).

Meteorological conditions represent a key determinant of drift, particularly wind (Albaseer et al. 2025). Under ‘good agricultural practice’, CUP application is therefore recommended only under stable weather conditions, with temperatures not exceeding 25 °C, relative humidity above 30% and wind speeds below 5 m/s (Landwirtschaftskammer Nordrhein-Westfalen 2023). To minimise overspray and drift, regulatory bodies recommend the use of approved border nozzles or switching off the outer nozzles at the field edge (Federal Ministry of Food and Agriculture 2017). The application in our drift study was performed under these best circumstances, resulting in low drift in the first 20 m adjacent to the field (Table 2, Paper 3). According to the benchmark values used in pesticide authorisation, less than 2.77% of the applied amount remains in 90% of the cases at 1 m distance from the field edge of field crops and with increasing distances drifted proportion decline (Julius Kühn-Institut 2022).

While drift deposition measured in Petri dishes placed at 1, 5 and 20 m distances to the field remained within or below regulatory benchmark values (Ganzelmeier et al. 1995; Rautmann et al. 2001; Julius Kühn-Institut 2022), soil and vegetation samples at the same distances frequently showed higher and more variable drift deposition (in 19 out of 45 cases, Table 2). This discrepancy indicates that simplified drift collectors may underestimate actual exposure in real environmental matrices. It also suggests that drift values used in the regulation only apply to the best-case scenario. In reality, these best-case conditions cannot be assumed to occur during all application events. This suggests that benchmark values may not always be sufficiently conservative.

Table 2 Drift deposition [%] based on the measured concentrations of the applied CUPs (Pirimicarb: Pir, Bixafen: Bix, Fluopyram: Flu, Prothioconazole: Pro) and a metabolite of Prothioconazole, Prothioconazole-desthio (PD), in bare, Petri dishes and soils and vegetation at the three meadows adjacent to the fields. Bold italics indicating a higher value than expected by the benchmark values (1m: 2.77%, 5m: 0.57%, 20m: 0.15%, "benchmark values [%]"). Empty cells indicate no detections.

Matrix	Meadow	distance	benchmark values [%]	Pir [%]	Bix [%]	Flu [%]	Pro [%]	PD [%]
Petri dish	1	1		0.01				0.05
Petri dish	2	1	2.77	0.03			0.03	0.02
Petri dish	3	1		0.04				0.05
Petri dish	1	5		0.01				
Petri dish	2	5	0.57	0.01				
Petri dish	3	5		0.01				
Petri dish	1	20		0.002				
Petri dish	2	20	0.15	0.002				
Petri dish	3	20		0.005				
Bare soil	1	1		0.26		0.11		
Bare soil	2	1	2.77	0.37	1.27	***3.42***		0.73
Bare soil	3	1			0.53	0.26		0.76
Bare soil	1	5		0.19		0.08		
Bare soil	2	5	0.57			0.21		
Bare soil	3	5				***0.65***		
Bare soil	1	20						
Bare soil	2	20	0.15			***0.16***		
Bare soil	3	20				0.15		
Soil + Vegetation	1	1		***3.60***		0.56		2.17
Soil + Vegetation	2	1	2.77	***3.17***	2.66	***10.46***		2.65
Soil + Vegetation	3	1		***5.42***	1.44	***4.65***		0.56
Soil + Vegetation	1	5		***4.57***		***0.58***		***1.17***
Soil + Vegetation	2	5	0.57	***1.21***		***0.91***		***0.63***
Soil + Vegetation	3	5		0.18		***0.54***		
Soil + Vegetation	1	20		***0.80***		***0.48***		***0.53***
Soil + Vegetation	2	20	0.15	***0.33***		***0.39***		
Soil + Vegetation	3	20		***0.47***		***0.27***		

Although all CUPs were applied simultaneously, bixafen residues were detected in environmental matrices at a distance of 1 m from the field after application. In contrast, pirimicarb was detected up to distances of 20 m. These differences likely reflect compound-specific physicochemical properties influencing drift potential and environmental transport. Vegetation plays an important role in intercepting drifting pesticide droplets and particles before they reach the soil surface. Consequently, drift deposition can be reduced in vegetation-covered soils compared with bare topsoil. At the same time, vegetation can act as an efficient collector of drifting CUP residues, leading to higher concentrations in vegetation samples than in soil. This pattern is consistent with observations from the year-long monitoring study (Paper 4), where residues assumed to originate from recent applications

were at higher concentrations in vegetation than in topsoil (see Table 4). Comparable patterns were also observed in the landscape-scale monitoring study (Paper 5), which is discussed in the following sections.

Secondary drift has been linked to the soil type that affects volatilisation and particle transport via wind (Albaseer et al. 2025). CUPs bound to soil particles can be transported by wind and enter the atmosphere. Long-range transport of CUPs in the atmosphere is a result of secondary drift and can carry CUPs far beyond their application areas (Mayer et al. 2024). This atmospheric transport may also explain the detections of CUPs that had not been applied during the study in environmental samples. However, it cannot be distinguished whether CUP residues in the fields and adjacent meadows may also represent residues of previous applications and their drift. These findings underscore that off-field exposure is not driven solely by single application events but rather by cumulative, overlapping CUP inputs.

5.2 Persistence of CUPs in Terrestrial Matrices

Dissipation of the applied CUPs from the drift study (Paper 3) was observed to be faster than predicted by literature-based DT_{50} values and a dissipation model called MITAS (Sybertz et al. 2020) in in-field topsoil samples. Dissipation is a key process determining the environmental presence of CUPs within a system and is comprised of degradative and non-degradative processes. Among the influencing factors are various abiotic factors, such as application parameters, CUP properties and environmental conditions, as well as biotic factors, including plants and soil microorganisms (Fenner et al. 2013; Farha et al. 2016). Dissipation is also influenced by non-degradative losses, such as volatilisation, leaching, and plant uptake (Vagi and Petsas 2021). On the other side, degradation describes the biotic transformation processes of pesticides mediated by microorganisms and plants, as well as abiotic processes such as chemical and photochemical reactions (Fenner et al. 2013). Soil properties further modulate dissipation and degradation processes. Soil humidity, soil type, organic matter content, and pH affect biological activity and chemical processes, thereby playing a central role in controlling CUP dissipation in soil (Rodríguez-Cruz et al. 2006; Borowik and Wyszkowska 2016). These factors and higher initial concentrations following direct application (Paper 3) may stimulate microbial degradation, resulting in faster initial dissipation, while the resulting low concentrations were not fully dissipated. Differences between the calculated and the reported DT_{50} values may also be a result of the fact that dissipation rates used in regulatory risk assessment are typically determined from laboratory studies under standardised conditions (Ranke et al. 2021) or semi-field studies and that for regulatory purposes, only degradation within the soil is considered, while other loss processes are excluded.

While dissipation data for soil are widely available, comparable data for vegetation are scarce (Fantke et al. 2014). Dissipation of CUPs in plants involves processes similar to those in soils, including abiotic and biotic transformation (Fantke and Juraske 2013), but also dilution due to plant growth. In vegetation, dissipation rates were generally consistent with literature values, indicating a relatively rapid decline of residues following application. In contrast to the in-field data, dissipation in non-target samples was substance-specific and often slower than in-field dissipation or could not be clearly quantified due to low and heterogeneous concentrations. For some substances, residues persisted in non-target vegetation and topsoil for extended periods, indicating prolonged exposure potential for non-target organisms in adjacent habitats. In line with these findings, on a larger temporal scale, CUP residues have been detected in fields and in adjacent non-target areas (Paper 4, Figure 10). The number of CUP residues in topsoils remained almost constant throughout the year, in-field, as well as in adjacent non-target areas. An average of 18 CUPs has been detected in agricultural topsoils, with many CUP concentrations in the µg/kg range, assuming those to originate, for example, from previous applications. This suggestion is confirmed by another study, which showed that pesticide residues can be detected in agricultural soils long after their initial application (Riedo et al. 2023). This is in line with an Europe-wide study that investigated CUP residues in fields and compared the results with application data (Knuth et al. 2024). This study revealed that 79% of the detected CUPs had not been applied in the current season (Knuth et al. 2024). Complex and varying CUP mixtures can be detected in agricultural soils worldwide (Tang and Maggi 2021). In contrast, little is known about the presence of CUP residues in vegetation. In vegetation sampled in the year-round study (Paper 4), temporal patterns revealed several maxima of the number of CUPs during the main application times, while CUP numbers in topsoils remained nearly constant (Figure 10). Vegetation is more exposed to CUPs than soils, as most of the CUPs are intentionally applied to plants. Moreover, plants intercept CUPs from atmospheric transport and take up systemic CUPs from the soil. Additionally, some crops are planted with seed coatings containing CUPs. Seed coatings are often not recognised as pesticide input (Hitaj et al. 2020), although they can contribute to the input of CUPs into the terrestrial system, including plants and soils.

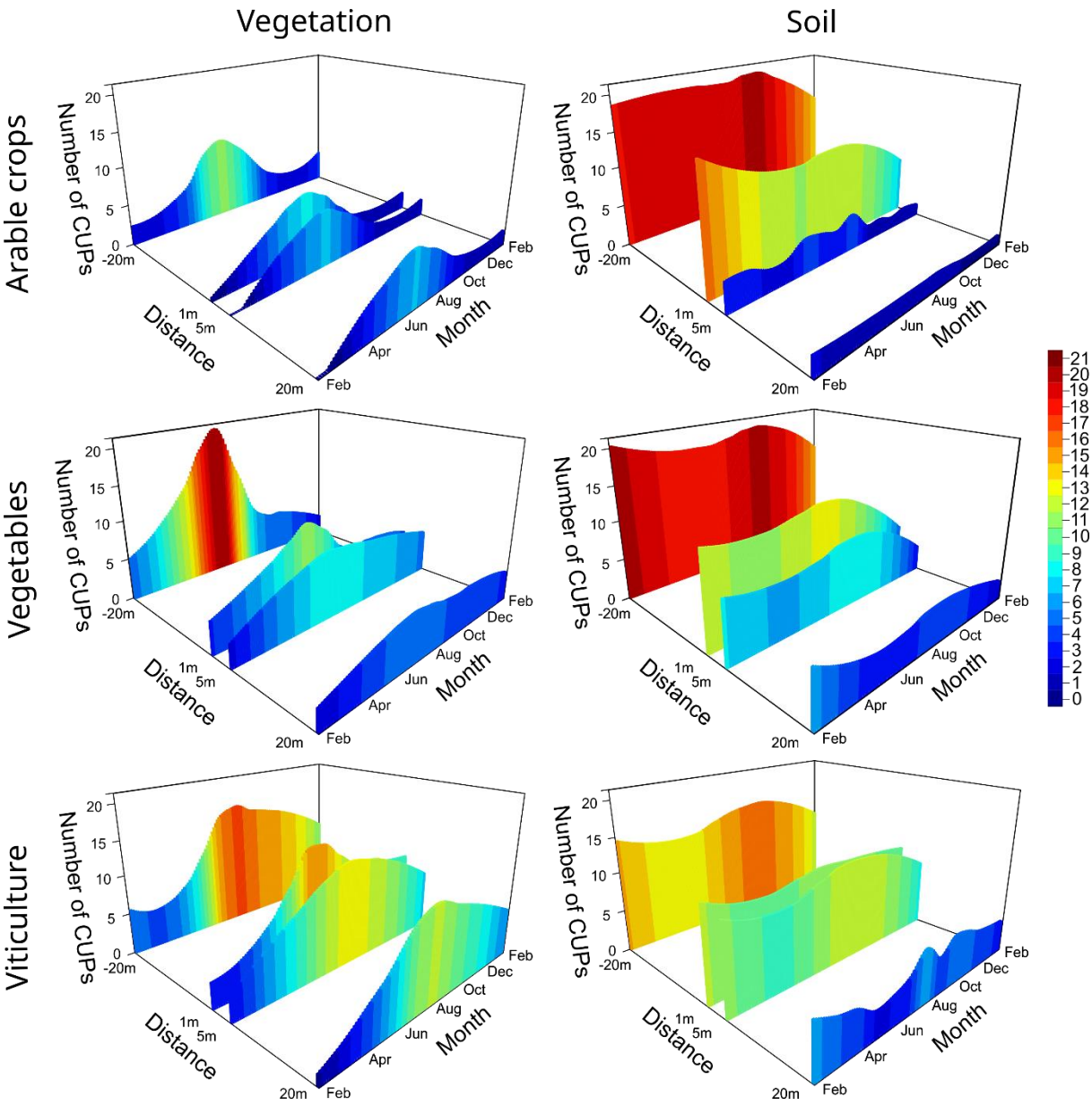


Figure 10 Number of CUPs throughout the year (months) in the field (-20m) and at different distances from the field edge (1, 5, and 20 m into the adjacent area) across arable, vegetable, and viticulture sites (vegetation left, soil right). Figure taken from Paper 4.

The pattern of the total number of CUPs over time was found to be influenced by cultivation type (Paper 4). However, the high number of detected CUPs suggests additional inputs beyond local applications. This indicates that CUP occurrence is also influenced by inputs from the surrounding agricultural areas, for example, through secondary drift and deposition. These processes not only affect target areas, but also lead to the occurrence of CUPs in non-target areas. Accordingly, CUP mixtures were recorded at 1, 5 and 20 m adjacent to the field year-round, with all topsoil samples containing at least one CUP (Paper 4). In a few vegetation samples (n=14), no CUP residue was detected; however, in 97% of the vegetation samples, CUPs were recorded (Paper 4). The off-field pattern of the number of CUPs followed the in-field pattern; however, the numbers decreased with increasing distance, consistent with the drift assessment results (Paper 3). Compositionally, the

detected residues were dominated by fungicides such as azoxystrobin and fluopyram, while herbicides and insecticides contributed less to the total number of CUPs. Although the measured concentrations of individual residues decline rapidly in in-field samples, repeated inputs and overlapping applications create a fluctuating but chronically present CUP pattern, which can be observed not only in agricultural fields, but also in adjacent non-target areas. It must be assumed that worldwide, not only agricultural soils contain CUP mixtures to certain sampling times, but that these mixtures are constantly present throughout the year and can also be detected in other terrestrial matrices and in the proximity to agricultural fields.

5.3 Spatial Structuring of CUPs

As CUP mixtures were detected throughout the year in non-target areas adjacent to agricultural fields, extending the spatial scale was a logical next step. Indeed, the landscape-scale investigation confirmed the widespread presence of CUPs in agriculturally dominated non-target areas (Paper 5). In summer months, CUPs were detected in 97% of both topsoil (n = 78) and vegetation (n = 78) samples. These findings complement the year-long study, where CUPs were present in 100% of the topsoil samples (n = 468) and 97% of the vegetation samples (n = 442), as well as the environmental-fate study, where CUPs were detected in all topsoil samples (n = 162) and in 97% of the vegetation samples (n = 93). Similarly, in the methods development (Paper 1), where in-field samples were analysed to validate the analytical method, CUPs were detected in 85% of topsoil samples collected across Germany (n = 13) and in all corresponding in-field vegetation samples (n = 13). In the landscape-scale study, CUP residues were also investigated in small surface water bodies. The detection of CUPs in 83% (n = 30) of the surface water samples demonstrated that CUPs are not confined to individual terrestrial matrices but are redistributed across interconnected environmental matrices.

To further characterise the spatial dimension of contamination, detection results from the landscape-scale study were extrapolated using spatial prediction models. The resulting predictions illustrate that CUP mixtures occur throughout the ~ 11,000 km^2 agriculturally dominated landscape, including areas distant from direct application sites (Figure 11). This spatial continuity highlights that exposure is not limited to field proximity but extends across broader landscape contexts. The observed patterns are consistent with findings from other European agricultural regions. In South Tyrol, an area strongly dominated by viticulture and orchards, at least one pesticide residue was detected in 96% in vegetation from public areas across all seasons of the year (Linhart et al. 2021). Similarly, CUPs were detected in 98% of vegetation samples and 59% of topsoil samples collected from remote areas during the main application period (Brühl et al. 2024). Taken together, these results demonstrate that the presence of CUPs is a persistent and spatially extensive feature of agricultural landscapes rather than an exception or local phenomenon.

Across all studies included in this thesis, fungicides constituted the dominant group of detected CUPs in both soil and vegetation. Their dominant market share in Europe may explain the frequent detection of fungicides (Research and Markets 2014), but also by the dominance of fungicides in the target list and is consistent with findings from other studies investigating terrestrial environments, including French soils (Froger et al. 2023) and Italian vegetation (Linhart et al. 2021). At the European level, herbicides have been reported as the dominant group in soil samples (Silva et al. 2019; Hvězdová et al. 2018) and represent the second most abundant in the studies included in this thesis. Insecticides are generally less present in agriculturally dominated landscapes. In contrast to fungicides and herbicides, insecticides are often used in smaller quantities per hectare. This results in a lower abundance in the environment. The predominance of fungicides reflects regional cropping systems and application practices. The Upper Rhine Valley is strongly influenced by viticulture growing, where fungicide use accounts for more than 90% of all CUP applications (Roßberg 2009). In viticulture, three-dimensional spray applications result in higher drift potentials compared to arable crops (Rautmann et al. 2001), as droplets are dispersed not only downward but also laterally and upward. This was reflected in the spatial distribution patterns observed during the year-long study, where CUP concentrations and mixture complexity decreased less rapidly with increasing distance from viticulture fields than from arable fields, leading to higher CUP detections in adjacent non-target areas (Figure 10). The annual average number of CUPs detected in topsoil at viticulture sites declined from 15 in-field to 12 at both 1 and 5 m distances and to 5 CUPs at 20 m from the field edge. In contrast, arable crop sites showed a steeper decline, with an average of 19 CUPs in-field, 13 at 1 m, 3 at 5 m and 2 at 20 m (Figure 10). Similar trends were observed in vegetation samples. Although the highest numbers of CUPs in non-target areas were measured at viticulture sites, arable crops exhibited the highest number of CUPs in the field. This likely reflects differences in application scenarios, as vineyards are treated repeatedly with a relatively small number of fungicides, whereas arable systems are characterised by crop rotation and changing products, resulting in a broader spectrum of CUP mixtures.

The importance of crop type for the spatial distribution of CUPs in surrounding non-target areas was also evident in the environmental samples of the landscape-scale study and the models underlying the landscape-scale prediction maps. Based on detections of CUPs in non-target vegetation, a maximum of 18 CUPs was predicted for viticulture-dominated landscapes. Overall, a higher diversity of CUPs was detected in the Upper Rhine Valley, with 63 CUPs, than in a comparable study in the Vinschgau in South Tyrol, where a total of 27 CUPs was recorded (Brühl et al. 2024). This difference may reflect crop-specific CUP authorisations and contrasting cropping systems between the regions, with viticulture in the Upper Rhine Valley and apple production in the Vinschgau.

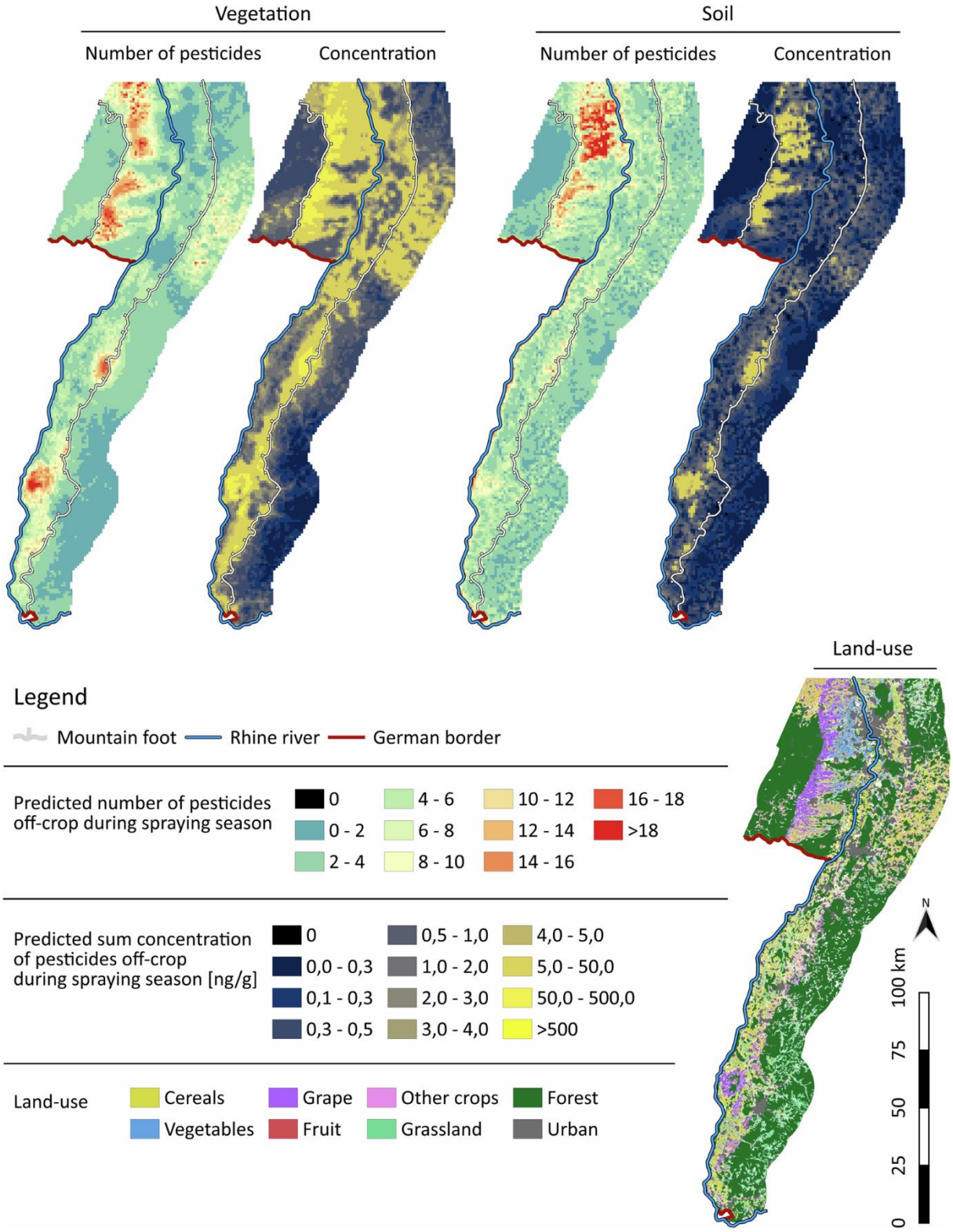


Figure 11 The in number of CUPs and sum concentration ng g^{-1} in off-field vegetation and topsoil during the spraying season were predicted with land-use categories in a linear model (CUP sum concentration) or generalised linear model (CUP number). Multiple R^2 is between 0.62 and 0.76. The raster pixel size is 1 × 1 km. Forest/urban land cover data are from the European Environment Agency (EEA) (2016); the remaining land use categories are from Blickensdörfer et al. (2021). The prediction map is based on the topsoil and vegetation measurements at 78 sites. Figure taken from Paper 5.

The spatial analyses demonstrate that CUP mixtures in topsoils and vegetation are a pervasive characteristic of agricultural landscapes and therefore result in ongoing CUP exposure for insects associated with topsoil and vegetation. For FVI, exposure is additionally strongly affected by CUPs entering pollination-related matrices and is integrated into resources actively used during feeding, reproduction or development. FVI interact directly with multiple environmental matrices, which, besides vegetation, also include flowers and pollen, and may therefore experience exposure across space and time.

5.4 Flower-Visiting Insects' Exposure to CUPs

FVI are potentially exposed to CUPs from multiple terrestrial matrices through their foraging and nesting activities. In contrast to soil- or vegetation-associated insects, their exposure is strongly shaped by the availability and CUP presence in floral resources, pollen and nesting materials, as well as by spatial foraging behaviour.

CUP mixtures were detected in all matrices that are particularly relevant for FVI (Paper 2). In flowers sampled between March and June, CUPs were consistently detected in 100% of the samples (n = 20, Table 4). This confirms that flowers located near agricultural fields represent an important exposure pathway, even though they are not directly targeted by CUP applications. Similarly, CUPs were frequently recorded in pollen provision sampled from *O. bicornis* nesting aids. CUPs were detected in 94% of the pollen provision samples (n = 35). Differences in CUP contamination between flowers and pollen suggest that pollen exposure is not solely determined by the presence of CUPs in nearby floral resources. Female *O. bicornis* may forage on floral resources located further away (Ziółkowska et al. 2023), particularly in landscapes with heterogeneous flower availability, thereby integrating CUP exposure over a larger spatial scale.

Insights into the spatial integration of exposure were further assessed through analysis of pollen collected from *A. mellifera* (Paper 6). Although CUP detections in pollen collected by *A. mellifera* increased with the proportion of annual cropland at the sampling site, overall, only 39% of the samples (n = 72) contained detectable CUP residues. Compared to topsoil, vegetation, flowers and *O. bicornis* pollen provision, this represents a remarkably low detection frequency (Table 4). *A. mellifera* preferentially exploit large and mass-flowering resources and are known to forage over distances of up to several kilometres (Danner et al. 2016). Consequently, *A. mellifera*-collected pollen reflects exposure at a broader landscape scale rather than contamination in proximity to treated fields. A high proportion of sunflower pollen was positively associated with the probability of fungicide detections and higher fungicide concentrations. Nonetheless, no clear association was found between fungicide detections and fungicide applications in sunflower crops. *A. mellifera-collected* pollen was sampled in

late summer, beyond the time at which the highest CUP residues in pollen are typically reported. Previous studies from Germany and other European regions have shown that CUP concentrations and pollen diversity both peak during spring and early summer, while markedly lower levels are observed in late summer (Böhme et al. 2018; Friedle et al. 2021; Murcia-Morales et al. 2021). Several important application events, such as aphid control or seed treatments during the early stages of crop establishment, were not covered during the sampling period. Nonetheless, pronounced temporal peaks occurred, with maximum residues several times higher than those recorded during the six days before and after. These findings underline a strong seasonal variability of CUP exposure in pollen of *A. mellifera* and demonstrate that exposure of honey bee pollen is highly time-dependent.

In 39% of post-pupal *O. bicornis* individuals that developed from pollen provisions collected during the early summer months, CUPs were detected, although they had never left their brood chamber. CUPs were detected in 39% of the analysed post-pupal *O. bicornis* individuals (n = 33) that hatched exclusively under laboratory conditions. As these individuals were only exposed to their brood chambers, the most plausible exposure routes were the pollen provision and the nesting material. Females use surrounding soil to separate brood cells, and this soil likely originated from adjacent meadows or fields, where CUPs were consistently detected (Paper 4). During insect metamorphosis, fundamental changes in body chemistry and tissue organisation occur (Kraus et al. 2014). These processes may contribute to the elimination of contaminants, for example, through excretion pathways associated with exuviae and meconium (Kraus et al. 2014; Wan et al. 2025a). However, the extent to which these processes reduce CUPs in developing bees remains largely unexplored. The lower number of CUPs detected in post-pupal bees compared with pollen provisions (Table 4) may therefore partly reflect detoxification and excretion processes occurring during metamorphosis. Nevertheless, CUP residues were still detected in post-pupal individuals. Pendimethalin was the most frequently detected compound, occurring in 28% of all analysed post-pupal bees. This herbicide is widely detected in the atmosphere (Kruse-Plaß et al. 2021) and is characterised by a lipophilicity (Giglio et al. 2019), which facilitates its association with organic matrices and may contribute to its persistence in biological tissues. Pendimethalin has also been shown to cause sublethal effects in insects (Giglio et al. 2019; Vommaro et al. 2021). The detection of CUP residues in post-pupal bees demonstrates that CUP exposure can occur even before adult emergence, highlighting CUP-containing pollen provisions and nesting materials as important and previously underappreciated exposure pathways. These findings further suggest that CUP residues may be transferred across developmental stages and through CUP-containing food resources across trophic levels.

Overall, the consistent detection of CUP mixtures in FVI matrices demonstrates that exposure is not restricted to environmental matrices but extends into biological systems. While matrix-specific target

lists and analytical sensitivity influence absolute detection frequencies and mixture sizes, CUP residues were detected across all investigated environmental matrices, including topsoil, vegetation, surface water, flowers, pollen provision and insects (Table 4). This widespread occurrence highlights the potential for continuous exposure of non-target insects and emphasises the relevance of these environmental pathways for subsequent risk assessment.

Maximal number of CUPs in single samples differed across environmental matrices, with surface waters exhibiting the greatest diversity, followed by soils, vegetation, flowers and pollen provisions (Table 4). Within the environmental fate study, a single topsoil sample contained 42 CUPs, the highest number of CUPs per sample overall (Table 4). Such complex mixtures indicate a diverse exposure scenario for FVI, which interact daily with multiple environmental resources. In addition, high summed concentrations measured in the environmental matrices (Table 4), further suggest that current exposure scenarios may underestimate the actual environmental concentrations encountered by insects. The steady, simultaneous exposure to complex CUP mixtures across multiple pathways is currently inadequately addressed in environmental risk assessment and remains insufficiently understood. Therefore, further research is needed to characterise this lifelong exposure of non-target insects better.

Table 3 Overview over the main results of CUP residues in environmental samples (topsoil, vegetation, surface water, flowers, pollen provision collected by wild bees and honey bees and newly hatched wild bees) collected and analysed in the complementary studies (Papers 2 – 6) included in this thesis. Number of samples, detection frequency [%], mean and maximal numbers of CUPs per sample, maximal sum concentration [µg/kg for terrestrial samples and µg/L for surface water], as well as the most frequently detected CUP (F: fungicide, H: herbicide and detection frequency [%]) per matrix and study.

Matrix	Study	Number of samples	Detection frequency [%]	Mean number of CUPs	Maximal number of CUPs	Maximal sum concentration	Most frequently detected CUP
Topsoil 	Year-round study (Paper 4)	468	100	10	28	2,594.71	Fluopyram (F, 92%)
	Environmental fate study (Paper 3)	162	100	22	42	458.07	Fluopyram (F, 100%)
	Landscape-scale (Paper 5)	78	97	5	26	248.23	Fluopyram (F, 94%)
Vegetation 	Year-round study (Paper 4)	442	97	7	25	91,554.49	Fluopyram (F, 72%)
	Environmental fate study (Paper 3)	93	100	7	16	1,855.54	Terbuthylazine (H, 99%)
	Landscape-scale (Paper 5)	78	97	6	21	1,966.51	Fluopyram (F, 92%)
Surface water 	Landscape-scale (Paper 5)	30	83	10	39	17.41	Fluopyram (F, 77%)
Flowers 	Method-Paper (Paper 2)	20	100	10	15	14,218.44	Terbuthylazine (H, 74%)
Pollen provision *O. bicornis* 	Method-Paper (Paper 2)	35	94	5	15	4,101.20	Terbuthylazine (H, 56%)
Pollen *A. mellifera* 	*A. mellifera*-study (Paper 6)	72	39	1	5	3,580.16	Azoxystrobin (F, 25%)
Newly hatched *O. bicornis* 	Method-Paper (Paper 2)	33	39	1	2	1,567.18	Pendimethalin (H, 28%)

6 Risks for Non-Target Organisms (Objective 3)

The following chapters evaluate the potential risks posed by detected current-use pesticides (CUPs) to non-target organisms across different terrestrial environmental matrices. The assessment builds on the risk analysis presented in Paper 3, while results from Papers 2–6 are integrated into the discussion.

6.1 Conceptual Limitations of Current Risk Assessment

The complementary studies revealed an exceptionally high level of complexity in CUP mixtures in agricultural-dominated landscapes. In the year-long study, 349 unique CUP mixtures were detected in 468 topsoil samples, and 344 unique mixtures were identified in 442 vegetation samples. At the landscape scale, 140 unique mixtures were observed across 78 sites. This pronounced high variability of CUP mixtures demonstrates that environmental exposure does not occur as isolated single substances, but as highly variable combinations.

Such complexity is not reflected in current regulatory risk assessment frameworks. Standard authorisation procedures primarily evaluate active substances individually and only consider predefined tank mixtures explicitly proposed. Sequential CUP applications (Frische et al. 2014; Sybertz et al. 2020), landscape-level accumulation and the co-occurrence of residues from previous seasons or neighbouring fields are not systematically addressed. Moreover, co-formulants contained in commercial products are often not publicly disclosed and are typically excluded from ecotoxicological assessment, despite evidence that they may modify toxicity profiles (Straw and Brown 2021). Given that our analytical methods covered only a subset of authorised CUPs in Germany, the actual environmental mixture complexity is likely even greater than reported here.

In addition, substantial taxonomic gaps persist in regulatory datasets. Regulatory pesticide risk assessments are typically based on a limited number of standardised model species such as *A. mellifera* or the earthworm *Eisenia fetida* (Wan et al. 2025b; Jegede et al. 2026). While these organisms provide valuable and reproducible test systems, their representativeness for the broader diversity of terrestrial insects is limited. Earthworms, although ecologically important soil organisms, are not insects and differ fundamentally in physiology, exposure routes and detoxification mechanisms. Extrapolation from earthworm toxicity data to soil-dwelling insects, such as ground-nesting wild bees or beetle larvae, is therefore associated with considerable uncertainty and may not be adequately covered by assessment factors. Honey bees, in contrast, are insects, but their social organisation, foraging range, and pollen-collecting strategies differ substantially from those of solitary bees and many other pollinators. As demonstrated in Papers 2 and 6, differences in foraging behaviour and time, as well as resource use, can result in markedly different exposure patterns, even within similar landscapes.

Moreover, species-specific responses to field-realistic CUP exposure have been documented (Jütte et al. 2023), indicating that sensitivity cannot be reliably generalised across taxa. The limited transferability of toxicity data has been highlighted in several recent publications (Raine and Rundlöf 2024; Pohl et al. 2024).

Moreover, the publicly available toxicology data basis does not include toxic endpoints for soil insects, despite soil representing a major habitat for a wide range of insects (Anthony et al. 2023). Some species live permanently underground, such as certain ants. In contrast, many others spend part of their life cycle in the soil, including larvae of the Dune chafer *(Anomala dubia)* and various Diptera species. Ground-nesting wild bees, particularly within the diverse genus *Andrena* (mining bees) and some *Bombus* (bumblebee) species, are likewise directly exposed to CUPs in soils during nest construction and larval development or hibernation. Nevertheless, soil insects are not explicitly represented in standard regulatory assessments. Our monitoring data demonstrate the absence of CUP-free periods in agriculturally dominated landscapes. Residues were detected throughout the year in topsoil, indicating continuous exposure for soil-dwelling insects and for developmental stages of insects such as larvae and pupae. Under such conditions, recovery processes based on temporary relief from exposure appear unlikely.

Similarly, herbivorous insects are not systematically addressed in pesticide risk assessment (Umweltbundesamt 2026) and no oral toxicity endpoints are required for foliage-feeding non-target insects such as beetles or grasshoppers. Consequently, risks arising from the consumption of vegetation containing CUP mixtures cannot currently be quantified within existing regulatory frameworks. This limitation is particularly relevant given that vegetation in our studies frequently contained higher CUP concentrations than topsoil samples, not only immediately after application (Paper 3), but also throughout the year-round study (Paper 4) and at the landscape scale (Paper 5). In addition, insecticides were detected in vegetation at all off-field distances adjacent to arable and vegetable crops, whereas they were absent in off-field topsoil samples. These findings indicate that exposure via plant material may represent an important exposure pathway for many insect taxa.

Beyond direct feeding exposure, vegetation plays a central ecological role for numerous insects. Many herbivorous species hibernate on host plants during their larval stages, while vegetation also provides oviposition sites, habitat and winter shelter. For example, adult butterflies such as the common brimstone (*Gonepteryx rhamni*) overwinter in vegetation, and many species hibernate as pupae on their larval host plants. Consequently, pesticide contamination of vegetation may affect insects across multiple life stages. High-resolution sampling further revealed pronounced seasonal dynamics of CUP residues in vegetation. The maximum number of CUPs was recorded during summer months, coinciding with peak larval feeding activity and oviposition of many phytophagous insects. Exposure

during these sensitive developmental stages has been shown to result in measurable fitness impairments. For example, larvae exposed to pesticide residues on host plants exhibited reduced longevity and altered wing morphology in emerging butterflies (Olaya-Arenas et al. 2020). Insecticide-treated plants have also been associated with reduced pollination and constrained oviposition behaviour in moths, leading to a 40% reduction in offspring (Hahn et al. 2015). Similarly, sublethal exposure of adult wild bee *O. cornuta* to field-realistic mixtures containing only a fungicide and an insecticide resulted in reduced reproductive output and subsequent population decline (Stuligross and Williams 2020; Albacete et al. 2024). Carryover effects across life stages have been documented in wild bees (Stuligross and Williams 2021) and other non-target insects (Müller 2018), indicating that even transient exposure events may alter population dynamics. Field observations linking pesticide exposure to mortality events in monarch butterflies at overwintering sites (Cibotti et al. 2025) further illustrate that exposure pathways may extend beyond agricultural fields and affect insects in their habitats. Residues of CUP mixtures have been documented in natural butterfly habitats and have been discussed as a major factor in the decline of the Apollo butterfly (Parnassius apollo) (Buijs and Mantingh 2020). Among the compounds discussed in this context is the fungicide fluopyram (Laußmann et al. 2025).

The substance-specific properties of CUPs could further strengthen these concerns. Fluopyram was not only the most frequently detected fungicide across most of the studies in this thesis (Table 4), but was also detected in every investigated environmental matrix, including topsoil, vegetation, flowers, pollen provisions, and surface water. Fluopyram is not only a fungicide, but is also sold as a nematicide (Schleker et al. 2022) and its metabolite has caused deformation in plants (Robatscher et al. 2019). Moreover, fluopyram is a polyfluorinated substance that is part of the per- and polyfluoroalkyl substances (PFAS), a group of chemicals characterised by high environmental persistence. Approximately 12% of the synthetic CUPs currently authorised in the European Union fall into the PFAS category (Pesticide Action Network and générations Futures 2023). Denmark banned the use of PFAS-pesticides as the first country in the EU and proposed, with other European national authorities, a restriction of PFAS (Sonne et al. 2023). The consistent detection of fluopyram across environmental matrices highlights the need to evaluate the risks posed by PFAS-pesticides and further demonstrates a structural regulatory gap regarding persistent CUPs.

A similar discrepancy between regulatory evaluation and environmental reality is evident for prosulfocarb, the herbicide most frequently detected in vegetation samples in the year-long study. Prosulfocarb was initially classified as posing a high risk to the parasitoid wasp *Aphidius rhopalosiphi*, a standard non-target arthropod test species (EFSA 2007). However, the overall risk was ultimately considered low due to assumptions of rapid degradation and recovery of field populations. This

recovery assumption has been criticised by the German Federal Environment Agency (Umweltbundesamt 2024b). Given the persistent and repeated detection of prosulfocarb residues in both agricultural and adjacent habitats, including insect samples from conservation areas (Brühl et al. 2021), rapid population recovery may be unlikely under realistic field conditions.

Importantly, regulatory exposure assessments are typically based on single application events and assume temporal regeneration between treatments. In contrast, the monitoring data presented here indicate that organisms in agricultural landscapes may experience continuous background exposure throughout the year. The continuous exposure to low concentrations may impair recovery processes and increase the likelihood of sublethal or chronic effects at the population level.

Together, these findings indicate that current pesticide risk assessment does not sufficiently capture the ecological reality of chronic, mixture-based exposure across multiple environmental matrices. The detected residue patterns suggest continuous background exposure rather than isolated peak events. Under such conditions, assumptions of population recovery and compartmentalised exposure likely underestimate risks to non-target organisms in agricultural landscapes.

6.2 Exposure-Based Mixture Risk Calculation

Over the one-year monitoring period, cumulative risks arising from measured soil concentrations were not confined to a single month but occurred repeatedly throughout the year (Table 5). Among the investigated organisms, earthworms showed the highest frequency of elevated cumulative risk quotients, followed by springtails and wild bees. In several sampling events, the calculated mixture risk was high, even though individual substances were present at low concentrations well below their respective regulatory trigger values. This indicates that additive effects of multiple residues can generate ecologically relevant risks that would not be detected in single-substance evaluations. This assumption is supported by a unique laboratory study that tested the effects of a CUP mixture at environmentally realistic concentrations on Diptera and observed pervasive sublethal effects even at shorter exposure durations. Interactions between many combinations of widely detected CUPs suggested widespread synergistic effects (Gandara et al. 2024). It can therefore be expected that sublethal effects can also occur at low environmental concentrations, similar to those measured in the presented studies.

Fungicides contributed substantially to cumulative risk, despite often being regarded as comparatively low-risk for non-target insects. This is particularly alarming, due to growing evidence that fungicides enhance the toxicity of other pesticide classes through synergistic interactions, particularly with neonicotinoids and other systemic CUPs (Sgolastra et al. 2017; Raimets et al. 2018; Schuhmann et al. 2021).

Risk levels varied among crop types, reflecting differences in application intensity and residue types. Notably, cumulative high risks were observed even in off-field soils, demonstrating that exposure is not confined to treated crop areas. These findings align with studies from other European regions reporting mixture-related risks in agricultural soils and adjacent habitats (Vašíčková et al. 2019)

Taken together, the exposure-based mixture assessment indicates that risks for soil organisms are likely to be underestimated under current regulatory paradigms if only single substances are considered. The results highlight the need to incorporate mixture-based and landscape-level approaches into future pesticide risk evaluation.

Table 4 Maximum Risk Quotients for Collembola and earthworms and maximum hazard quotients calculated for wild bees based on the measured environmental concentrations (MECs) and toxicological endpoints of CUPs in topsoils in cultivated areas (in-field) of arable (n=3), vegetable (n=3) and viticulture sites (n=3). The maximum quotient at each cultivated site and month is shown. No risk (white) MRQ > 0.01, low risk (light grey) 0.01 < MRQ < 0.1, medium risk (grey) 0.1 < MRQ < 1, very high risk (black) MRQ > 1. Table adapted to Paper 4.

	Feb'21	Mar'21	Apr'21	May'21	Jun'21	Jul'21	Aug'21	Sep'21	Oct'21	Nov'21	Dec'21	Jan'22	Feb'22
Arable crop													
Collembola	0.51	0.82	0.83	0.64	1.02	2.46	1.08	1.75	1.49	0.49	0.66	0.81	0.3
Earthworm	0.43	0.44	0.73	0.54	1.82	0.46	0.32	0.42	0.45	0.51	0.35	0.33	0.17
Wild bee	0.49	1.13	1.80	1.19	3.9	4.54	0.94	0.71	0.74	0.52	1.28	0.68	0.01
Vegetable fields													
Collembola	0.43	0.30	1.17	0.33	0.85	0.32	0.47	0.59	3.2	2.19	0.9	0.69	0.86
Earthworm	0.31	0.60	0.70	1.02	2.03	7.33	0.62	1.43	4.23	1.95	1.18	0.86	1.00
Wild bee	2.08	0.01	0.52	0.37	0.12	0.66	1.91	5.65	22.69	14.82	3.93	3.64	4.09
Viticulture													
Collembola	0.05	0.09	0.25	0.31	2.3	2.87	6.61	0.83	1.12	0.66	0.8	0.3	0.21
Earthworm	0.39	3.83	1.44	1.52	2.03	2.86	5.02	1.75	1.31	1.07	1.94	0.86	0.69
Wild bee	0.05	0.18	0.07	0.06	0.12	0.15	0.29	0.10	0.09	0.06	0.12	0.05	0.05

6.3 Implications for Protected Areas and Landscape-Level Effects

The results of this thesis demonstrate that CUP exposure extends beyond agricultural fields and applications affecting entire landscapes, including protected areas. Nature conservation areas are generally assumed to provide refuge for non-target organisms. However, the present findings challenge this assumption. Model predictions revealed a mean of 4-5 CUPs in vegetation and 5-6 CUPs in topsoils within nature conservation areas. In viticulture-dominated regions such as the Kaiserstuhl, where 16 small-sized conservation areas are embedded within intensive agricultural landscapes, maximum values of up to 20 CUPs in vegetation were predicted. These predictions align with detections from the conservation area “Kleine Kalmit”, which is surrounded by viticulture, where 16 CUPs were detected during the main application time in the year-long monitoring study. Exposure in protected areas is therefore strongly influenced by the surrounding landscape. A correlation analysis revealed the strongest relationship between the surrounding arable area and CUP numbers recorded

for insects caught in nature conservation areas within a 2 km radius (Brühl et al. 2021). This indicates that effective protection of insects cannot rely solely on the designation of protected areas but requires reductions in CUP use in the surrounding agricultural landscape. Establishing sufficiently large buffer zones with zero-pesticide use around conservation areas may therefore represent an important strategy to reduce CUP exposure of non-target organisms.

The observed continuous exposure across environmental matrices challenges the assumption that populations in treated fields can recover through recolonisation from nearby refuges. As CUPs occur across the wider landscape, potential source populations are also exposed and may be affected by CUPs. Such landscape-level exposure patterns are consistent with observations of insect declines in agriculturally dominated regions. For instance, long-term studies in south-western Germany, including the Upper Rhine Valley, revealed particularly strong declines in macro-moth diversity and abundance in nature reserves situated in regions characterised by intensive agricultural land use (Habel et al. 2026).

From a policy perspective, these findings are relevant in light of the precautionary principle, which serves as a guiding principle in German, European, and international environmental policies. The precautionary principle states that regulatory action does not require complete scientific certainty but may be justified when there are reasonable grounds for concern (Umweltbundesamt 2024a). As the ecological effects of complex CUP mixtures across different environmental matrices remain insufficiently understood, precautionary measures to reduce CUP risks appear justified. These concerns are addressed by several policy initiatives. For example, the German National Action Plan for the Sustainable Use of Plant Protection Products aims to reduce risks associated with CUP use (German Ministry of Food and Agriculture 2023). Furthermore, European policy initiatives such as the Nature Restoration Law aims to expand sustainable agriculture, including the expansion of organic farming systems and moderate CUP reduction (European Parliament 2024). Such approaches could contribute to reducing CUP exposure to non-target organisms and support the recovery of ecosystem functions and services in agricultural landscapes. On the other hand, the failure on the Sustainable Use Regulation (SUR) at the EU level highlights ongoing challenges in translating scientific evidence into regulatory action, and the rejection of further negotiations poses a significant challenge to environmental protection.

Overall, increasing scientific evidence indicates that current pesticide use patterns and existing risk assessment frameworks may not sufficiently protect biodiversity. Numerous studies have linked pesticide use to declines in insect populations and broader biodiversity loss (Geiger et al. 2010; Dudley et al. 2017; Brühl and Zaller 2019; Solé et al. 2024). A link between the observed decline in insect biomass (Hallmann et al. 2017), the decline in biodiversity and the use of pesticides is considered

proven (Geiger et al. 2010; Liess et al. 2021; Dicks et al. 2021). Current risk assessments, which largely rely on simplified exposure scenarios and single-substance evaluations, therefore underestimate the ecological risks posed by CUP mixtures and landscape-scale exposure patterns. Addressing these challenges will require a transition towards system-based risk assessment approaches that integrate spatial and temporal dynamics of pesticide exposure (Topping et al. 2020).

7 Conclusion and Outlook

In this thesis, the application of complementary, matrix-adapted multiresidue methods enabled the characterisation of current-use pesticides (CUPs) across terrestrial environmental matrices. The analytical framework developed in this work enabled the detection of complex residue mixtures in soils, vegetation, flowers, pollen provision, and insects at environmentally realistic concentrations. The set of methods provides a transferable framework for further terrestrial monitoring. The methods were applied in this thesis to investigate the environmental fate, the occurrence, distribution and temporal dynamics of CUPs in environmental matrices relevant to insects. To our knowledge, this thesis provides one of the most comprehensive assessments of spatial and temporal gradients of pesticide residues across terrestrial environmental matrices to date, with more than 1,000 environmental samples. By analysing a large number of CUPs across multiple matrices with high temporal resolution, the study enables direct comparison of CUP occurrence across habitats and seasons. This approach allows CUP exposure to be evaluated not only in terms of occurrence but also in relation to potential ecological risks. Such integrative assessments remain rare for terrestrial ecosystems and provide an important step towards understanding the real-world exposure scenarios experienced by non-target insects.

The results consistently show that modern agricultural landscapes are characterised by the ubiquitous presence of CUP mixtures. Even under best-case application scenarios, drift into adjacent non-target areas was observed. The discrepancy between standardised drift collectors, here Petri dishes and environmental matrices, indicates that current regulatory drift assessments underestimate actual exposure in terrestrial ecosystems, as they focus primarily on primary drift and simplified deposition scenarios. Dissipation investigations revealed that CUP residues dissipated fast in the field, but tended to persist beyond their expected dissipation time in non-target areas. Within the high-resolution monitoring approach over one year, CUP mixtures were detected throughout the whole year in multiple environmental matrices in agriculturally dominated landscapes, including vegetation and soils. In particular, the number of CUPs in vegetation exhibited pronounced seasonal exposure peaks coinciding with periods of high insect activity, whereas the number of CUPs in soils remained constant throughout the year. Such chronic, temporally structured exposure patterns are not explicitly incorporated into current regulatory environmental risk assessment, highlighting the importance of considering mixture exposure in ecological risk assessment.

Furthermore, the presence of CUP mixtures was not confined to agricultural fields and nearby adjacent areas but extended across the landscape. Landscape-scale analyses revealed the widespread occurrence of complex mixtures, including areas designated for nature conservation. These findings

challenge assumptions underlying recovery concepts in regulatory risk assessment, which often rely on recolonisation from non-target areas. It further calls into question the function of protected areas as effective refuges for insects in intensively managed landscapes. Besides soils and vegetation, CUP residues are also present in flowers and pollen provision for *O. bicornis* larvae, revealing CUP exposure throughout different environmental matrices. The detection of residues in newly emerged individuals right after leaving their brood chamber further indicates that pesticide exposure can permeate multiple biological levels and life stages.

Taken together, this thesis points out substantial limitations of current pesticide risk assessment approaches, which are largely based on single-substance evaluations and simplified exposure scenarios (Figure 12). These findings support previous critiques that current risk assessment is insufficiently addressing ecological complexity and realistic exposure scenarios (Uhl and Brühl 2019; Brühl and Zaller 2019; Topping et al. 2020). The complex and continuous CUP exposure patterns observed in this thesis emphasise the need for assessment frameworks that reflect real-world conditions and incorporate mixture toxicity, landscape-scale exposure and several environmental matrices. Moreover, assessments focusing on a limited number of surrogate species do not adequately capture potentially endangered insects, particularly soil-dwelling insects, herbivorous larvae, and ground-nesting wild bees.

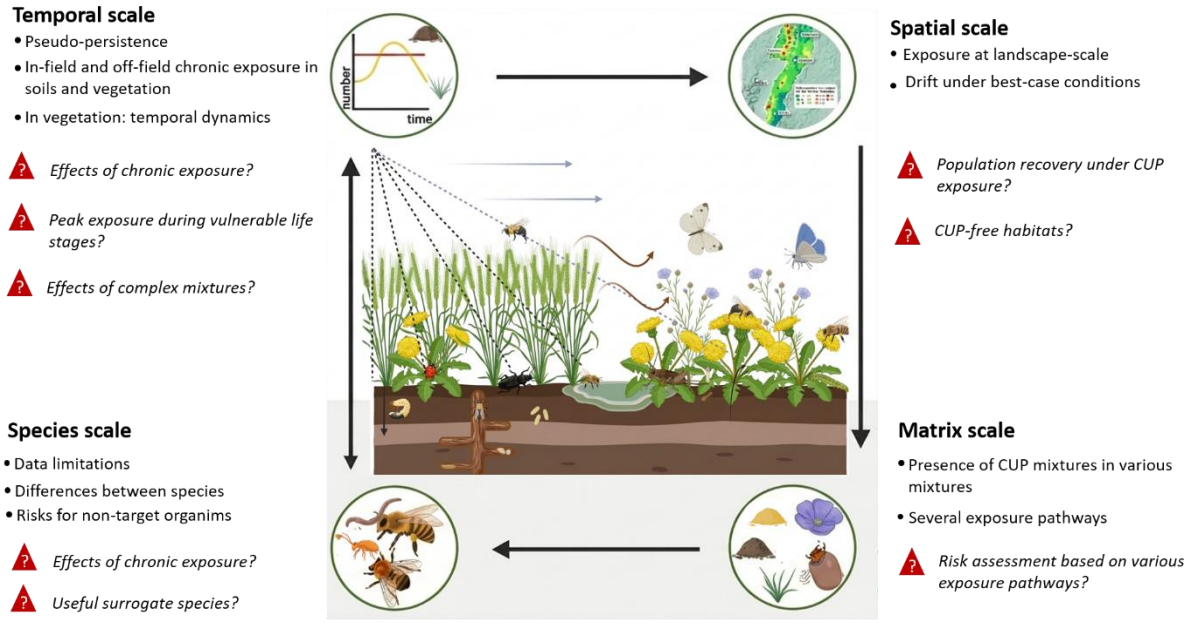


Figure 12 Synthesis of the key findings in this thesis and the occurring open questions (italics) leading to the assumption that the current authorisation procedure of CUPs fails to reflect real-world conditions. Created with biorender.com.

In the context of the documented declines in insect biodiversity within the study region (Habel et al. 2026) as well as worldwide (Wagner et al. 2021; van Klink et al. 2024; van Klink et al. 2020), the findings contribute empirical exposure data to the ongoing scientific debate on the role of CUPs for biodiversity

loss. While causality between observed population declines and specific exposure patterns cannot be established within the scope of this thesis, the documented spatial ubiquity, mixture complexity and chronic nature of CUP contamination underline the ecological relevance of CUP exposure in agriculturally dominated landscapes. Under the precautionary principle, which forms a cornerstone of European environmental policy (European Commission 2000), persistent uncertainty regarding mixture effects and chronic exposure, CUP use should be reduced or stopped until further scientific evidence is available to verify its safety for non-target insects. In agricultural areas adjacent to protected areas, a substantial reduction of CUP use appears necessary to limit the ongoing input of biologically active substances with largely unknown ecological effects. More broadly, reducing overall CUP use is an important step towards reducing environmental contamination. Alternative agricultural practices, such as permaculture, have been proposed as ecologically sustainable approaches to mitigate biodiversity loss (Reiff et al. 2024) while maintaining yields comparable to those of conventional agriculture (Reiff et al. 2025). However, the implementation of such approaches is constrained by established socio-technical systems and economic interests that shape current agricultural practices and policies (Goulet et al. 2023). These conditions contribute to the continued exposure of organisms, not only insects but also other non-target species, including humans, to a wide range of CUP mixtures. Given the substantial uncertainties regarding the effects of chronic mixture exposure, reducing CUP inputs into the environment appears advisable. Effectively mitigating the risks posed by CUPs to insects, humans, and planetary health (Vandenberg et al. 2025) is likely required to science-based policies that extend beyond current regulatory and economic frameworks.

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9 Author contributions

This is a cumulative dissertation containing five articles published in peer-reviewed international journals and one submitted manuscript:

Paper 1 (Appendix I):

Nikita Bakanov: Conceptualization, Methodology, Validation, Investigation, Writing – Original Draft; **Carolina Honert**: Investigation, Writing – Review & Editing; **Lisa Eichler**: Software, Writing – Review & Editing; **Gerlind U.C. Lehmann**: Project administration, Writing – Re view & Editing; **Ralf Schulz**: Writing – Review & Editing; **Carsten A. Brühl**: Supervision, Writing – Review & Editing.

Paper 2 (Appendix II):

Carolina Honert wrote the main manuscript text, coordinated the logistics, was responsible for project administration and data curation and arranged the necessary permissions from relevant authorities. **Carolina Honert, Katharina Wifling. and María José Lazó Hernandez** collected the samples, performed the analytical analysis and prepared the data. **Carolina Honert** and **Katharina Wifling.** analysed the data statistically and produced the figures. **Carsten A. Brühl** planned, acquired funding, supervised the entire project and selected CUPs for analysis. All the authors discussed the results, commented on and improved the manuscript and agreed on the final version.

Paper 3 (Appendix III):

Carolina Honert wrote the main manuscript text, coordinated the logistics, was responsible for project administration and data curation and arranged the permissions. **Carolina Honert** and **Ursel Jäger** took the samples, performed the analytical analysis and prepared the data. **Carolina Honert** performed the statistical analysis and produced the relevant figures. **Alexandra Sybertz** implemented and applied the model for predicting time-dependent soil pesticide concentrations and reviewed and edited the original draft. **Martina Roß-Nickoll** contributed to the model implementation and application for dissipation prediction and reviewed and edited the original draft. **Carsten A. Brühl** developed the study idea, acquired funding, supervised the entire project, participated during application and sampling and reviewed and edited the original draft. **Carolina Honert, Ursel Jäger., Carsten A. Brühl** and **Alexandra Sybertz** discussed the results. All the authors commented and improved the manuscript and agreed on the final version.

Paper 4 (Appendix IV):

Carolina Honert: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Ken M. Mauser**: Methodology, Formal analysis, Data curation, Writing – review & editing. **Ursel Jäger**: Methodology, Formal analysis, Writing – review & editing. **Carsten A. Brühl**: Conceptualization, Methodology, Resources, Investigation, Writing – review & editing, Project administration, Supervision.

Paper 5 (Appendix V):

Ken M. Mauser: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Carolina Honert**: Methodology, Formal analysis, Data curation, Writing – review & editing. **Jakob Wolfram**: Methodology, Formal analysis, Writing – review & editing. **Jürg W. Spaak**: Methodology, Formal analysis, Writing – review & editing. **Carsten A. Brühl**: Conceptualization, Methodology, Resources, Investigation, Writing – review & editing, Project administration, Supervision

Paper 6 (Appendix VI):

Ingolf Steffan-Dewenter: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Ricarda Scheiner:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Giulia Mainardi:** Writing – review & editing, Investigation. **Carsten A. Brühl:** Writing – review & editing, Resources. **Carolina Honert:** Writing – review & editing, Validation, Methodology. **Alexander Keller:** Writing – review & editing, Methodology, Formal analysis. **Douglas Sponsler:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Sarah Manzer:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis.

10 Appendices

Appendix I: Paper 1

A new sample preparation approach for the analysis of 98 current-use pesticides in soil and herbaceous vegetation using HPLC-MS/MS in combination with an acetonitrile-based extraction

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A new sample preparation approach for the analysis of 98 current-use pesticides in soil and herbaceous vegetation using HPLC-MS/MS in combination with an acetonitrile-based extraction

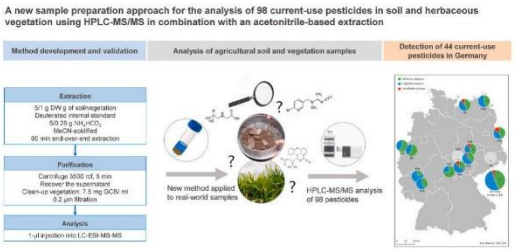
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HIGHLIGHTS

- Simple method for extraction and analysis of 98 pesticides in soil and vegetation.
- First study on pesticide load of agricultural soils and vegetation in Germany.
- More than 50% of pesticides detected in soil were linked to applications in the previous years.

GRAPHICAL ABSTRACT



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ABSTRACT

A simple acetonitrile-based extraction method for the determination of 98 current-use pesticides (CUPs) in soil and herbaceous vegetation using HPLC-ESI-MS/MS is reported. The method was optimized in terms of extraction time, buffer (ammonium formate) ratio, and graphitized carbon black (GCB) ratio for the clean-up of vegetation. The validated method yielded accuracy in terms of percentage recovery of 71–125% (soil) and 70–117% (vegetation) for the majority of 98 CUPs. The precision in terms of relative standard deviation was at 1–14% (soil), and 1–13% (vegetation). Matrix-matched calibration curves exhibited good linearities ($R^2 > 0.99$). The limits of quantitation ranged between 0.008 and 21.5 μg kg^{-1} in soil and vegetation. The reported method was applied to soils and vegetation from 13 agricultural sites across Germany. Overall, 44 of the 98 common CUPs were detected in our samples and the qualitative load is well above the average for arable soils in the EU.

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1. Introduction

Vital agricultural soils are key for the continuation of human civilization since nearly 99% of the daily calories consumed by humans come from the cultivation of crops (FAO and ITPS, 2018). Industrial agriculture heavily relies on synthetic pesticides (Tilman et al., 2002) to control yield losses to weeds, pathogens, and pests (Sharma et al., 2019). In the European Union (EU), approximately 451 active pesticide substances are approved on the market for commercial plant protection products (EC, 2022) and 346 × 10^3 tonnes are applied on agricultural soils annually (EUROSTAT, 2022). When pesticides reach the soil compartment, they affect earthworms, microbiota, and processes such as soil nitrification and respiration (soil health), all of which are addressed by the EU environmental risk assessment procedures for each pesticide during its application for commercial/industrial use. Multiple pesticides are used in application sequences on crops, resulting in mixtures of residues present in soils. The first global data on pesticide residues in soil were published recently (summarised in Sabzevari and Hofman, 2022). For Germany, to the best of our knowledge there is no data available, yet a recent study highlighted that changes in pesticide use in Germany have led to risks to soil organisms increasing over time (Bub et al., 2023).

To evaluate pesticide contamination of agricultural fields and other terrestrial habitats, a monitoring approach, like those established for surface waters for decades, needs to be implemented. To tackle this task, pesticide analysis should be established at reasonable costs and high sample throughput at the same time. The extraction of pesticides from solid matrices such as soil and vegetation plays a key part in the sample pre-treatment process as it requires labor time, many methodological steps, and often relatively high volumes of solvents, making the pre-treatment process an expensive and potentially error-prone procedure. Recently published methods for the analysis of pesticide residues in soil are based on adapted versions of the "Quick, Easy, Cheap, Effective, Rugged, and Safe" (QuEChERS; Anastassiades et al., 2003) methodology (Geissen et al., 2021; Kosubova et al., 2020; Silva et al., 2019; Hvezdova et al., 2018). QuEChERS is currently the most commonly used sample preparation approach in pesticide residue analysis and has evolved from an unbuffered version (Anastassiades et al., 2003) to an acetate buffering (AOAC Official Method 2007.01) or citrate buffering (CEN Standard Method EN 15662) version. QuEChERS comprises the extraction step, in which magnesium sulfate (Lehotay, 2007; Payá et al., 2007) in combination with sodium chloride, acetate, or citrate salts are used, followed by the clean-up step of the resulting supernatant using dispersive solid-phase extraction (dSPE).

Modifications of the QuEChERS methodology have been reported, aiming to address instrument performance losses in LC analysis, due to solid salts'/ions' deposits commonly used for extraction in QuEChERS such as magnesium sulfate in combination with sodium chloride, acetate, or citrate salts on the surface in the MS sources (González-Curbelo et al., 2014; Nanita and Padivitage, 2013). The authors reported ammonium formate (NH_4HCO_2) provided the best performance and practical considerations for the extraction of 43 representative pesticide analytes of different classes from a 15 g fresh weight sample in comparison to $NH_4HCO_2$and acetate buffers. González-Curbelo et al. (2014) used mostly GC-amenable pesticides in apple, lemon, lettuce, and cereals matrices to evaluate the performance of NH_4HCO_2. However, for a wider scope of monitoring of targeted pesticides in soil and vegetation sample matrices, this approach requires further evaluation.

Modifications of the QuEChERS methodology have been also applied to the agitation of the soil sample during extraction. Agitation protocols ranging from 1 min to 60 min shaking of the soil sample with acetonitrile (MeCN) were validated and published (Geissen et al., 2021; Kosubova et al., 2020; Silva et al., 2019; Hvezdova et al., 2018; Dankyi et al., 2014).

The central aim of this study was to develop an efficient method for the extraction and detection of 98 current-use pesticides (CUPs) in soil and vegetation samples, utilizing a simple approach with minimal components. MeCN was chosen as the extracting solvent, while formic acid and NH_4HCO_2 were used to enhance the extraction process. To determine the optimal parameters for the extraction of pesticides, we tested varying extraction times/sample agitation modes, NH_4HCO_2 ratios, and contact times of NH_4HCO_2 with the soil and vegetation matrices. In addition, we evaluated different amounts of graphitized carbon black (GCB) for an optimal purification of vegetation sample extracts. The developed method was validated and applied to the analysis of soil and vegetation samples obtained from 13 agricultural sites in Germany, using high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) to detect the presence of CUPs residues. The proposed method offers a streamlined and effective approach for the simultaneous extraction and detection of CUPs residues in soil and vegetation samples.

2. Material and methods

2.1. Analytical standards, chemicals, and reagents

The 98 pesticide reference standards (purity ≥98%) were purchased as multi-component solutions (concentration, 100 mg L^{-1}) from Restek (Bellefonte, PA, USA) or as single-component standards/solutions from either Dr. Ehrenstorfer (Augsburg, Germany) or Sigma-Aldrich (St Louis, MO, USA). An individual stock solution (100–400 mg L^{-1}) was prepared from each single-component standard using MeCN (LC-MS grade, Honeywell, Seelze, Germany). The individual stocks and the multi-component solutions were combined to make the final multi-residue mixed standard (5 mg L^{-1}) in MeCN, which was further diluted with MeCN to produce concentrations of 0.0005, 0.001, 0.01, 0.1, and 1 mg L^{-1}. All pesticide working solutions were stored at −20 °C.

For the preparation of buffered mobile phases, methanol (MeOH, LC-MS grade, Honeywell, Seelze, Germany), water (LC-MS grade, Merck, Darmstadt, Germany), formic acid (≥99%, VWR International, Leuven, Belgium), and NH_4HCO_2 (≥99%, Sigma-Aldrich, St Louis, MO, USA) were used. For extraction of samples, MeCN (HPLC-grade, Honeywell, Seelze, Germany) and ammonium formate (reagent grade, Sigma-Aldrich, St Louis MO, USA) were used. The filtration of samples was performed using 0.2 μm filters (17 mm HPLC syringe filter, PTFE, hydrophobic, BGB Analytik, Lörrach, Germany).

2.2. Analyzed CUPs

The list of analyzed CUPs was compiled based on records of the Julius-Kühn-Institute (JKI) published in the PAPA database (JKI, 2019). The JKI records were evaluated for the frequency of pesticides used in winter wheat, oilseed rape, maize, potato, and grapes in 2016 and 2017. The most frequently-used herbicides and fungicides as well as all insecticides were selected. The list was further supplemented with the biocide fipronil and its metabolites fipronil sulfone and fipronil desulfinyl, as well as pesticides regularly occurring in agricultural streams in Germany (Liess et al., 2021), and insecticides (3 CUPs) registered in the period of 2015–2016. Banned legacy pesticides such as DDT and pesticides that are no longer authorized for use in the European Union were not the focus of this study and were not included. In the final list of 98 analyzed CUPs, herbicides accounted for 38% (37) and fungicides for 37% (36); insecticides and biocides/biocide metabolites made up 22% (22) and 3% (3), respectively.

2.3. HPLC-ESI-MS/MS

Pesticide concentrations were measured using an HPLC-ESI-MS/MS system (HPLC: Agilent Technologies LC 1260 Infinity II series, MS/MS: Agilent Technologies 6495C, Santa Clara CA, USA). Details on the instrument method conditions are given in SOM Table S1 (HPLC) and Table S2 (MS/MS). The mass spectrometer was set to dynamic multiple reaction monitoring (dMRM) for selective detection of the analyzed

CUPs. The quantitation and confirmation of the CUPs were performed using at least two MRM transitions (except for proquinazid one MRM transition ion pair was used), the retention time (RT), and a signal-to-noise ratio (S/N) of >10 for the quantifier ion. Details on the MS/MS method were published by Brühl et al. (2021). In this work, we report the determination of pure biological active isomers dimethenamid-P, metolachlor-S, metalaxyl-M, and quizalofop-P. Apart from the active isomers, plant protection products also contain non-active isomers (e.g. metalaxyl as the seed treatment active ingredient agent is a 50:50 racemate of R- and S-isomers). Both the active and non-active isomers cannot be separated in reverse-phase chromatography. Therefore, the isomeric constituents of the active substance in plant protection products should be taken into consideration when the risk assessment is performed to avoid misinterpretation of the magnitude of adverse effects on non-target organisms.

Information on MS/MS parameters for all pesticide analytes of the present study can be found in Table S3. The HPLC-MS/MS data were processed using Agilent MassHunter Workstation (Quantitative analysis for QQQ version 10, Agilent Technologies, Inc., Santa Clara CA, USA).

The samples were quantified using matrix-matched external calibration (calibration standards, $n = 8$). For matrix-matched standards, blank soil and vegetation matrix (aliquots of the centrifuged, cleaned, and filtered liquid extract) was fortified with the multi-residue working solutions at the calibration concentrations of 0.025, 0.05, 0.1, 0.5, 1, 10, 50, and 100 μg L^{-1}. The calibration curve was then constructed by analyzing the matrix-matched standards. The use of matrix-matched calibration is a common approach to minimize the effects of the matrix on the measurement (i.e. enhancement or suppression of the ion intensity of targeted compounds), resulting in more accurate and precise analytical results (e.g. De Carlo et al., 2015).

To ensure continuous quality control, we employed the use of a deuterated analytical standard, imidacloprid-D4 (Dr. Ehrenstorfer, Augsburg, Germany), to monitor the extraction recovery of both field soil and vegetation samples. Prior to extraction, the soil and vegetation samples were fortified with imidacloprid-D4 (25 μl at 10 mg L^{-1}). The response of imidacloprid-D4 in both soil and vegetation samples was then compared to that of the respective blank soil or vegetation sample, which was fortified with imidacloprid-D4 after extraction. Please note that the deuterated standard imidacloprid-D4 was not used to correct the measured concentrations of the CUPs.

2.4. Samples and sample preparation

The samples of soil and vegetation used in this study were collected in May 2021 from 13 sampling sites located in agricultural fields. The samples were part of DINA project (Lehmann et al., 2021), which aimed to study the diversity of insects in various nature reserves in Germany adjacent to agricultural fields from 2019 to 2021. The sampling locations were selected to ensure a broad geographic representation.

Table 1 provides an overview of the agricultural practices employed at each site in 2020, including the type of crop planted, type of grazing, and the type of pesticide formulation applied to the fields. Although we do not have access to the precise timing of pesticide application in 2020 due to non-disclosure by farmers, we can infer that the applications occurred not later than 15 months prior to the collection of soil and vegetation samples, based on the types of crops grown. Table 2 presents a summary of the soil properties for the 13 sampling sites and the soil site used for method development and validation. The properties include texture, pH, organic carbon, nitrogen, C/N ratio, phosphate, potassium, magnesium, and organic matter.

Samples of soil and vegetation were collected from a rectangular area of 3.5 × 3.5 m, located 20 m from the edge of the field. Eight individual samples (sampling depth, 0–5 cm) were combined into a composite sample for each point of collection. After collection, the samples were stored in labeled plastic bags on cold packs and transported to the laboratory within 24–48 h. The soil samples were sieved (2 mm, ISO

Table 1
Sampling sites (= agricultural field) and type of agricultural use and pesticide formulations applied in 2020. Information was obtained from farmers. H: herbicide, F: fungicide, I: insecticide. †Pesticide not included in the analysis in this study. Pesticides in italics correspond to the CUPs applied in 2020.

Site	Crop/ Grazing	Applied pesticide formulation	Pesticides contained in the applied formulation	Overall pesticide detection in soil samples
RIE	winter wheat/ cattle	Battle Delta, Zensus, Fence, Tribun, Soleil, Teppeki	Diflufenican (H), flufenacet (H); tribenuron-methyl (F), bromuconazole† (F), tebuconazole (F), flonicamid (I)	*Diflufenican* (H), ethofumesate (H), *flufenacet* (H), metamitron (H), metazachlor (H), pendimethalin (H), propyzamide (H), prosulfocarb (H), azoxystrobin (F), bixafen (F), boscalid (F), epoxiconazole (F), fenpropimorph (F), metrafenone (F), pyraclostrobin, clothianidin (I), pirimicarb (I)
KOO	fallow/ cattle	no application of pesticides	–	Clomazone (H), diflufenican (H), flufenacet (H), flurtamone (H), metazachlor (H), pendimethalin (H), azoxystrobin (F), bixafen (F), dimethomorph (F), epoxiconazole (F), fenpropimorph (F), fluopyram (F), pyraclostrobin (F), tebuconazole (F)
MAL	winter wheat/ sheep, goats	Stomp Aqua, Agolin, Pointer SX, Bandur, Glyphosat 360	Diflufenican (H), pendimethalin (H), tribenuron-methyl (H), glyphosate† (H)	*Diflufenican* (H), chlorotoluron (H), dimethenamid-P (H), isoproturon (H), metazachlor (H), metolachlor-S (H), *pendimethalin* (H), terbuthylazine (H), azoxystrobin (F), bixafen (F), boscalid (F), epoxiconazole (F), metrafenone (F), spiroxamine (F), tebuconazole (F), acetamiprid (I), cyantraniliprole (I), thiacloprid (I)
WIS	corn/-	Successor T, MaisTer Power	Cyprosulfamide† (H), foramsulfuron (H), pethoxamide† (H), thiencarbazone-methyl† (H), terbuthylazine (H)	Diflufenican (H), flufenacet (H), *terbuthylazine* (H), bixafen (F), epoxiconazole (F)
BIS	corn/-	Herold, Input Triple, Ascra Xpro	Diflufenican (H), flufenacet (H), bixafen (F), fluopyram (F), spiroxamine (F), prothioconazole (F), proquinazid (F)	*Diflufenican* (H), isoproturon (H), terbuthylazine (H), *bixafen* (F), *fluopyram* (F)
POR	wheat/-	CTU, Pointer	Chlorotoluron (H), tribenuron (H)	*Chlorotoluron* (H), clomazone (H),

(*continued on next page*)

Table 1 (*continued*)

Site	Crop/ Grazing	Applied pesticide formulation	Pesticides contained in the applied formulation	Overall pesticide detection in soil samples
				diflufenican (H), flufenacet (H), metazachlor (H), metolachlor-S (H), napropamide (H), pendimethalin (H), propyzamide (H), quizalofop-P (H), terbuthylazine (H), azoxystrobin (F), bixafen (F), boscalid (F), dimethomorph (F), epoxiconazole (F), fludioxonil (F), fluopicolide (F), metconazole (F), spiroxamine (F), tebuconazole (F), clothianidin (I), imidacloprid (I)
ZIE	grassland, rye/-	no application of pesticides	–	–
WIP	grassland/-	no application of pesticides	–	–
BOT	winter barley/ sheep	no information available	Diflufenican (H), flufenacet (H), metsulfuron (H), azoxystrobin (F), chlorothalonil (F), pyrethroid (I)	Chlorotoluron (H), *diflufenican* (H), *flufenacet* (H), isoproturon (H), napropamide (H), pendimethalin (H), propyzamide (H), *azoxystrobin* (F), bixafen (F), boscalid (F), epoxiconazole (F), fluopicolide (F), fluopyram (F), myclobutanil (F), prochloraz (F), proquinazid (F), spiroxamine (F), tebuconazole (F), clothianidin (I)
SBG	barley, rye/-	no information available	no information available	Diflufenican (H), dimethenamid-P (H), flufenacet (H), metazachlor (H), pendimethalin (H), terbuthylazine (H), azoxystrobin (F), boscalid (F), difenoconazole (F), dimethomorph (F) dimoxystrobin (F), epoxiconazole (F) fludioxonil (F), fluopyram (F), metrafenone (F), pyraclostrobin (F), acetamiprid (I), clothianidin (I)
HOF	Fallow, clower, oats/shee	no information available	no information available	Diflufenican (H), isoproturon (H), metolachlor-S (H), pendimethalin (H), terbuthylazine (H), epoxiconazole (F), fluopyram (F)
KOP	–/–	no application of pesticides	–	Terbuthylazine (H), azoxystrobin (F), bixafen (F), epoxiconazole (F),
DOE	barley or rye/-	no information available	no information available	Diflufenican (H), isoproturon (H), metolachlor-S (H), bixafen (F), boscalid (F), dimoxystrobin (F), epoxiconazole (F), fluopyram (F), spiroxamine (F), tebuconazole (F)

RIE, Riedensee; KOO, Insel Koos; MAL, Oderhänge Mallnow; WIS, Wieseler Dünen; BIS, Bislicher Insel; POR, Porphyrlandschaft bei Gimritz; ZIE, Ziegenbuschhänge bei Oberau; WIP, Wipperdurchbruch; BOT, Bottendorfer Hügel; SBG, Schwellenburg; HOF, Hofberg; KOP, Koppelstein; DOE, Rheinhänge Dorscheider Heide.

3310–1/ASTM E11, Retsch, Haan, Germany) following their arrival at the laboratory. The samples were frozen at −20 °C until extraction and subsequent analysis. Blank soil and vegetation samples were collected from an area in Jockgrim, Germany (49°05′10.3″N 8°15′33.1″ E), where no agricultural land use was identified within a radius of 1 km for method development purposes.

2.5. Development of the extraction procedure

Three different agitation methods were used to investigate the effect of the extraction time on the extraction yields of the CUPs from soil. These included 1 min of hand shaking, a 60-min end-over-end agitation, and a combined approach of 1 min of hand shaking and 15 min of sonication, using MeCN as the extraction solvent. Although fortified samples are commonly used to assess the accuracy and precision of the analytical steps, they have limitations when it comes to measuring the efficiency (yield) of the extraction process, as they do not account for the binding of pesticides to the soil matrix. This can lead to inaccurate estimates of the amount of pesticide that is available for extraction. In this study, we employed an actual environmental sample taken from an agricultural field with a history of frequent pesticide use, to determine the extraction efficiency of the three protocols. After determining the optimal extraction time for maximum extraction yield, laboratory-prepared blank samples fortified with the 98 CUPs of interest were utilized to determine detection and quantification limits (LODs and LOQs) and recovery.

The HPLC-ESI-MS/MS analysis of the environmental sample employed to determine the extraction efficiency of the extraction protocols identified a total of 16 CUPs, comprising nine herbicides (chloridazon, diflufenican, dimethenamid-P, flufenacet, lenacil, metamitron, metolachlor-S, prosulfocarb, and terbuthylazine), four fungicides (azoxystrobin, epoxiconazole, ethofumesate, and fluopyram), and three insecticides (clothianidin, imidacloprid, and thiamethoxam). These CUPs belong to a variety of chemical classes, including triazines (chloridazon, terbuthylazine), phenylpyrazoles (ethofumesate), neonicotinoids (imidacloprid, clothianidin, thiamethoxam), azoles (epoxiconazole), carbamates (fluopyram), dinitroanilines (diflufenican, flufenacet, metolachlor-S), thiocarbamates (lenacil, prosulfocarb), triazinones (metamitron), chloroacetamides (dimethenamid-P), and strobilurins (azoxystrobin). Of these CUPs, seven were planar pesticides

Table 2
Summary of the soil properties for the 13 sampling sites and the soil site used for method development and validation.

Site	Soil texture	pH	Organic carbon (C) (%)	Nitrogen (N) (mg/100 g)	C/N ratio	Phosphate (mg/100 g)	Potassium (mg/100 g)	Magnesium (mg/100 g)	Organic matter (%)
RIE	ls	6.6	1.5	182	8.3	26	20	12	2.6
KOO	ls	6.1	0.9	99	9.4	12	13	5	1.6
MAL	wls	5.3	1.0	91	10.5	5	15	6	1.7
WIS	wls	5.0	0.9	95	9.1	16	11	5	1.5
BIS	lc	7.4	2.1	226	9.4	40	44	12	3.7
POR	wls	5.5	1.9	187	9.9	14	55	6	3.2
ZIE	cl	7.0	1.9	212	9.1	10	40	11	3.3
WIP	lc	7.4	3.6	358	9.9	70	27	21	6.1
BOT	ls	7.4	1.6	190	8.6	20	16	8	2.8
SBG	cl	7.7	1.4	177	7.9	21	33	16	2.4
HOF	cl	7.3	2.4	257	9.3	19	43	9	4.1
KOP	ls	7.3	2.3	248	9.1	29	72	9	3.9
DOE	ls	5.7	2.9	316	9.1	20	19	13	5.0
Jockgrim[a]	ls	5.8	2.6	214	8.5	18	21	15	2.4

Soil texture: ls: loamy sand, wls: weak loamy sand, lc: loess clay, cl: clay loam.
[a] Location of the soil's origin, which was utilized for the development and validation of the method Analysis performed at the Agricultural Investigation and Research Institute Speyer (LUFA Speyer).

(chloridazon, diflufenican, flufenacet, lenacil, metamitron, prosulfocarb, and terbuthylazine) detected in the environmental soil sample. In addition, the 16 CUPs exhibit a wide range of octanol-water partition coefficients (Kow) ranging from 0.9 to 4.2, indicating their ability to bind to soil matrices can vary from low to high. This feature makes them a suitable representative of pesticides with diverse binding potentials to soil.

After optimizing the extraction time, various ratios of NH_4HCO_2 (7.5 or 5 g of NH_4HCO_2 per 5 g soil sample) were used to investigate its impact on the extraction efficiency of the 98 CUPs from soil. Our primary objective was to enhance the extraction efficiency by optimizing the amount of NH_4HCO_2, instead of examining whether 7.5 or 5 g ammonium formate leads to instrument performance loss owing to solid deposit of the salt. Our assumption was based on the physicochemical properties of ammonium formate, which was experimentally demonstrated (González-Curbelo et al., 2014; Nanita and Padivitage, 2013) to cause fewer instrument performance losses than QuEChERS salts.

First, the 5-g soil sample was agitated with the previously determined optimal extraction time, and then NH_4HCO_2 was added to the sample and shaken vigorously for 1 min. Secondly, to evaluate the influence of the higher contact time with NH_4HCO_2 on the extraction efficiency, NH_4HCO_2 was added to the soil sample before being agitated for the optimal time.

The method optimization was extended to the vegetation matrix by including an additional step of purification using GCB, which is known to be effective in eliminating sterols and pigments such as chlorophyll that are often co-extracted in vegetable and plant matrices (Schenck et al., 2002). Chlorophyll is non-volatile material and can contaminate the LC-MS/MS source; negatively impacting the pesticide determination (it results in higher LOD). However, it has been reported that the application of GCB may negatively affect the accuracy of pesticide determination (Niessen et al., 2006; Wilkowska and Biziuk, 2011). GCB, other materials such as primary secondary amine (PSA), and C18 are widely (Wilkowska and Biziuk, 2011) used for clean-up for the removal of sugars, fatty acids, and lipids. But, as the herbaceous vegetation investigated in the present study did not contain any significant levels of lipids and fatty acids, PSA and C18 were not evaluated in the clean-up procedure. The clean-up efficiency of GCB was determined using blank samples fortified at pesticide concentration of 0.1 mg kg^{-1} and cleaned after extraction with 7.5, 10, or 20 mg of GCB.

2.6. Validation of the extraction method

The optimized extraction method was subjected to validation by analyzing several key parameters, including calibration linearity, matrix effects, detection and quantification limits (LODs and LOQs). Similar to previous studies on pesticides in soils (e.g. Panico et al., 2022; Geissen et al., 2021; Pelosi et al., 2021; Silva et al., 2019; Hvezdova et al., 2018), we directed our attention also towards the validation parameter recovery of the extraction method.

The LODs and LOQs were determined using blank samples fortified with pesticide solutions at concentrations ($n = 5$) corresponding to the signal-to-noise ratio of 10 of the respective CUP. The LOQ and LODs were calculated based on the standard deviations of the response and the slope of the matrix-matched calibration curve with seven calibration levels as LOD = 3.3 σ/S, LOQ = 10 σ/S, where σ is the standard deviation of the response, and S is the slope of the matrix-matched calibration curve.

The recovery was determined by analysis of blank samples fortified with standard solutions at three concentrations (0.01, 0.1, 0.25 mg kg^{-1}; $n = 7$). The recoveries were calculated as the ratio of the responses of the blank samples, which were fortified before extraction, to the responses of the blank samples, which were fortified after extraction.

Linearity was determined using matrix-matched calibration curves with fortified blank samples. The matrix effects (%ME) were calculated as the ratio of the slope of the matrix-matched pesticide calibration curve to the slope of the pesticide calibration curve in MeCN. An additional commonly used approach involves analyzing a blank matrix fortified at different concentration levels (such as LOQ, middle, and high concentration) to quantitatively assess the matrix effect. Both methods are equally effective in providing information on the presence and magnitude of matrix effects (Furey et al., 2013).

Continuous quality control was provided by monitoring the recoveries of the internal standard imidacloprid-D4 in soil and vegetation samples collected from 13 sampling sites. In case of a deviation of the recoveries of the internal standard by more than 20%, the extraction of the respective field soil or vegetation sample and the measurement of the pesticide concentrations were repeated.

3. Results and discussion

3.1. Method development

3.1.1. Optimization of the extraction time

The extraction of the 16 CUPs from the environmental soil sample was optimized using different agitation methods. The lowest extraction yields were obtained using the 1-min hand shaking method (Fig. 1). The extraction yields obtained from the 1-min hand shaking method were used as a reference to compare with the results of the other two agitation methods.

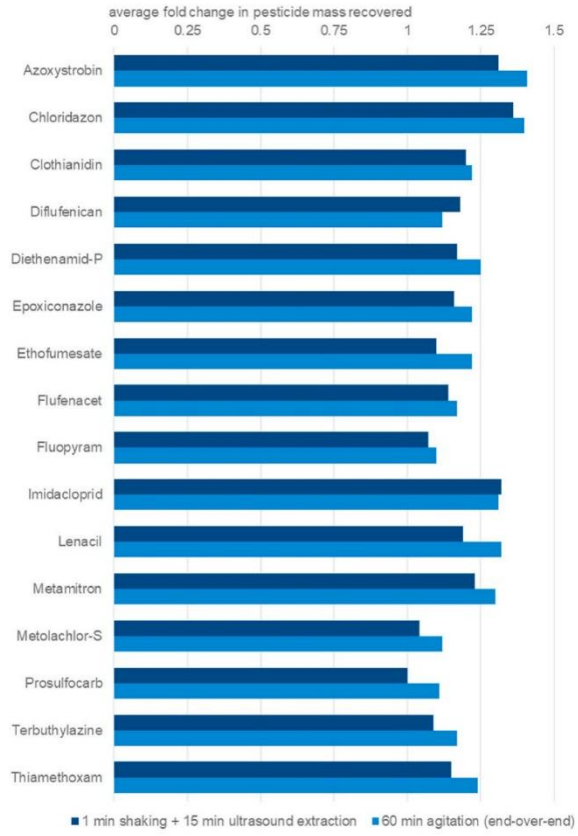


Fig. 1. Average (n = 3) fold change in pesticide mass recovered from the environmental soil sample using different agitation methods (combined approach of 1 min 15 min of sonication and 60 min of end-over-end agitation). Comparison is based on the mass recovered using 1 min of hand shaking as third agitation method.

The best extraction yields were achieved using the 60-min end-over-end agitation method (1.1–1.41-fold increase in recovered CUP mass) followed by the combined approach of 1 min of hand shaking and 15 min sonication (1.07–1.36-fold increase).

Judging from the extraction yields of the 16 CUPs present in the environmental soil sample and the different works previously published (Bragança et al., 2012), applying longer extraction times can lead to improved recovery values. The limitation of the QuEChERS approach for soil samples have already recognized by the inventors of QuEChERS (Lehotay et al., 2010b). While shaking has been shown to be an acceptable extraction method (QuEChERS) for incurred as well as spiked samples in food matrices, it may not be sufficient to overcome the binding characteristics present in real-world soils. In contrast to food matrices, these binding characteristics are much stronger in soils, which can negatively impact the effectiveness of shaking as an extraction method. The presented increased extraction yields observed with the addition of a sonication step in the extraction process support these arguments and are in agreement with previous research (Bragança et al., 2012; Fernandes et al., 2013).

Based on the results obtained, the 60-min end-over-end agitation method appears to be more suitable as it produced the best extraction yields (slightly higher compared to the combined approach of 1 min of hand shaking and 15 min sonication) and involved milder extraction conditions compared to ultrasonication. This is important because harsher extraction conditions can lead to increased extraction of soil matrix, which may negatively impact the accuracy of the analysis process.

3.1.2. Optimization of the NH_4HCO_2 ratio

When extracting with 7.5 g of NH_4HCO_2 per 5 g soil, 24% of all CUPs exhibited recoveries below 75%. A large proportion of the CUPs (75%) exhibited acceptable recoveries of 75–100% (Table S4). Recoveries in the range of 70–120% fulfill the acceptance criteria for regulatory methods (EC, 2005).

At 7.5 g of NH_4HCO_2, the solubility of the salt in MeCN was exceeded and some of the NH_4HCO_2 precipitated as a solid. It was therefore decided to reduce the amount of NH_4HCO_2 per g of soil to 1 g. The obtained recoveries were similar to those of the extraction with 1.5 g NH_4HCO_2: 9% of all CUPs exhibited recoveries of 0–25%, 3 and 13% recoveries of 25–50 and 50–75%, respectively. The recoveries were low particularly for quinmerac, picloram, clopyralid, fluroxypyr, 2,4-D, MCPA, propamocarb, quizalofop-P, pymetrozine, and foramsulfuron, exhibiting recoveries of 5–25%. However, the majority of the CUPs (75%) had acceptable recoveries of 75–100%. The reduced amount of NH_4HCO_2 of 1 g per g of soil provided high-quality results for 75% of all CUPs and was thus used for further optimization of the extraction method.

It was hypothesized that a relatively short contact time of 1 min with NH_4HCO_2 may limit the extraction efficiency for the CUPs, for which a low recovery was achieved (in total 25% of CUPs at recovery ranges of 0–75%). To test this hypothesis, NH_4HCO_2 was added to the sample before the 60- minutes end-over-end agitation. The addition of NH_4HCO_2 before extraction further improved the recoveries of 13% of the CUPs in the range of 10–29% (Table S5). However, seven CUPs still exhibited low recoveries, e.g. 2,4-D (recovery, 51%), fluroxypyr (36%), MCPA (64%), and picloram (10%). All of these CUPs are acidic, which necessitated the use of buffer a for the extraction. It was therefore decided to add 2.5% of formic acid added to the MeCN for extraction. Using acidified MeCN for extraction significantly improved the recoveries of the CUPs, for which low recoveries were obtained in non-acidified MeCN. For instance, 2,4-D had a recovery of 85% in acidified MeCN. Overall, the recoveries have been improved in the low-recoveries CUPs by 34–87% (Table S6), so that 100% of the 98 CUPs had an acceptable recovery.

3.1.3. Vegetation samples clean-up procedure

The content of the co-extracted pigments in the vegetation extracts was reduced for all three GCB amounts tested (Figure S2); the color of the cleaned extracts was light yellow as the result of the removal of chlorophyll compared to the dark green of the non-cleaned extracts. An amount of 7.5 mg mL^{-1} GCB was reported to remove 85–90% of the chlorophyll in the lettuces (Koesukwiwat et al., 2010). Recoveries for several planar CUPs were however negatively impacted, i.e. cyprodinil, pymetrozine, pyrimethanil, and quinmerac, which had recoveries of <70%. The low recoveries observed are consistent with literature reports on the interactions with GCB (Kaczyński and Łozowicka, 2017; Lehotay et al., 2010). When 20 mg of GCB was used, planar CUPs exhibited the lowest recoveries (40–70%). Applying only 10 or 7.5 mg of GCB for clean-up, recovery ranges increased to 59–79% and 70–97%, respectively (Figure S1). It is noteworthy that GCB had no negative effect on the planar herbicide terbuthylazine, which was at 84–87%. Also, for proquinazid, an acceptable recovery of 72% was demonstrated at 20-mg of GCB. It is conceivable that terbuthylazine and proquinazid are not among the CUPs having the highest affinity for the planar structure of GCB and therefore are not retained by the sorbent.

For remaining nonplanar CUPs, GCB was not observed to reduce the recoveries. Using the 7.5-mg GCB clean-up, the recoveries of 98 CUPs (100%) were at 75–109%, falling within the acceptance criteria for quantitative regulatory methods (EC, 2005). An amount of 7.5 mg GCB provided the best performance and practical considerations and was

therefore used for the clean-up of field vegetation samples. The clean-up procedure can/might be further evaluated by testing if lower amounts of GCB (e.g. 5 instead of 7.5 mg) would lead to similar valid results. However, our primary concern with the vegetation samples was the possibility of adverse matrix effects on the instrument caused by solid deposits in the source and ion transfer capillary. While 5 mg GCB might be adequate for effective purification of the extracts, we decided to use 7.5 mg GCB as a compromise to prevent long-term instrument performance losses.

3.2. Method validation

3.2.1. LOQ/LOD

In terms of soil analysis, the LOQs ranged from 0.008 (insecticide pirimicarb) to 19.1 (herbicide 2.4-D) μg kg^{-1} (Table S7). Of the CUPs analyzed in soil samples, 46, 43, and nine had LOQ values in the range of 0.01–0.1, 0.11–1.0, and 1.1–25 μg kg^{-1}, respectively. We conducted a comparison of the LOQs determined in this study with the LOQs reported in five recent studies that employed QuEChERS-based extraction methods (Panico et al., 2022; Hladik and Ward, 2022; Geissen et al., 2021; Pelosi et al., 2021; Silva et al., 2019; Hvezdova et al., 2018; Table S12). The number of CUPs common with our study varied from 15 (Panico et al., 2022) to 45 (Geissen et al., 2021) CUPs. Our study's LOQs were lower for 27–100% of the shared CUPs depending on the study.

For vegetation samples, the LOQ values for vegetation samples ranged from 0.01 (fungicide propamocarb) to 21.5 (herbicide picloram) μg kg^{-1} (Table S7). The distribution of LOQ values in the three concentration ranges (0.01–0.1, 0.11–1.0, and 1.1–25 μg kg^{-1}) was similar to that found for the soil samples (Table S7). While there has been a significant amount of research on the occurrence of pesticides in soils, little to no attention has been given to studying the presence of these compounds in wild and cultivated plants. Studies examining pesticide residues in plants have primarily focused on analyzing flowers (e.g. Hladik and Ward, 2022), as these plant parts are known to provide insects with nectar, honeydew, and pollen, making them more susceptible to pesticide contamination. The vegetation analyzed in the present study did not display significant blossoming, which presents a challenge when comparing the limit of quantifications (LOQs) or limit of detections (LODs) with the previously published research. Taking into account this constraint, when comparing the LODs between the present study and the cited studies, it was found that the shared 4–43 CUPs all exhibited lower LOD values in the present study (Table S13).

3.2.2. The recovery

In the validation of the recovery at the 0.01, 0.1, and 0.25 mg kg^{-1} fortification level, the CUPs showed good precision (percentage relative standard deviation, %RSD) (n = 7) of 1–14% in soil sample analyses (Table S8). Depending on the validation level, the CUPs exhibited recoveries at 71–125% for soil samples.

A similar good precision (n = 7) of 1–13% was obtained in vegetation sample analyses. Recoveries were at 70–117%, except for fludioxonil (recovery, 66%; RSD, 4%). At the 0.01 mg kg^{-1} fortification level, the calculation of the recovery was not applicable for 17 CUPs (herbicides, 2,4-D, aminopyralid, bromoxynil, carfentrazone-ethyl, fluroxypyr, MCPA, mecoprop, metsulfuron-methyl, picloram, quizalofop-P, tritosulfuron; fungicides, fludioxonil, pyrimethanil; insecticides, avermectin B1a, flonicamid, imidacloprid, pirimicarb), as the CUPs-signals were below the LOD. For 81 of the 98 CUPs, the obtained recoveries fulfilled the acceptance criteria for regulatory methods (EC, 2017; recovery range, 70–120%).

3.2.3. Linearity and matrix effect

The coefficients of determination of all 98 CUPs showed acceptable linearity (R^2 > 0.99, Table S9). The present study employed mild extraction conditions and used a clean-up sorbent GCB to minimize the amount of co-extracted substances from soil and vegetation samples that could potentially interfere with the CUPs, and thus, affect the accuracy of the targeted CUPs' response. For soil, responses of 97% (95) CUPs exhibited no ME (between −20 and 20%) (Table S10). The herbicide fluroxypyr and fungicide propamocarb showed medium-level enhancement effects (between 20 and 50%) and the herbicide tribenuron-methyl had a medium-level suppression effect (between −20 and −50%). For vegetation, 92% (90) of all CUPs exhibited no MEs, whereas 8% of tested CUPs (6 herbicides and 2 insecticides) showed medium-level enhancement effects (between 20 and 50%, Table S10).

3.3. Application of the validated method to field samples

3.3.1. Number of CUP residues detected

Out of the 98 CUPs investigated, 44 (45%) were detected in at least one of the soil samples, with an average of 11.2 CUPs per sample. In seven soil samples, the number of detected CUP residues was greater than the average detected in all 13 sites. At least five CUP residues were detected in 11 (85%) soil samples, with a maximum of 23 CUPs found in some samples (Fig. 2). However, two soil samples (ZIE and WIP) were found to be free of residues. According to the interview with farmers, no pesticides were utilized at ZIE and WIP (Table 1).

Out of the 98 CUPs, 13 (13%) were detected in at least one of the

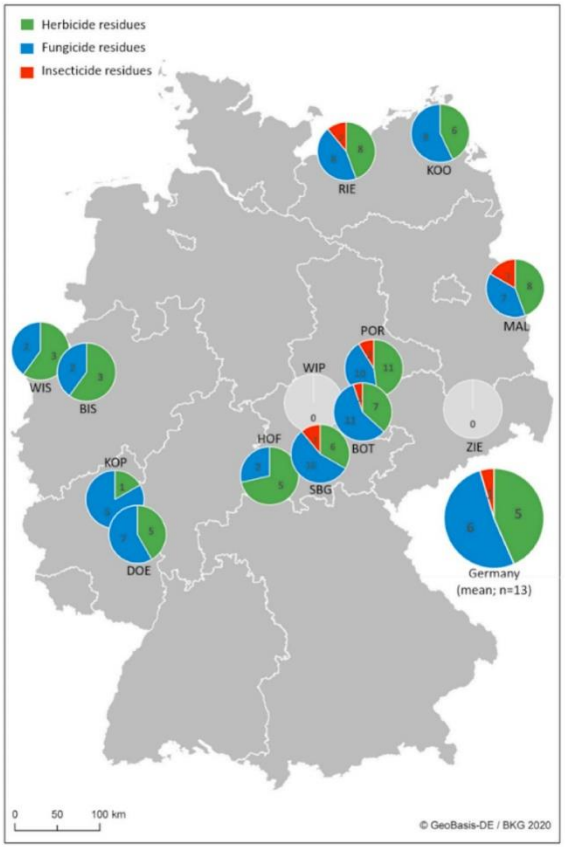


Fig. 2. Number of herbicides, fungicides, and insecticides per sampling site detected in the soil. For sampling location full names see Lehmann et al. (2021). Site abbreviations: RIE, Riedensee; KOO, Insel Koos; MAL, Oderhänge Mallnow; WIS, Wieseler Dünen; BIS, Bislicher Insel; POR, Porphyrlandschaft bei Gimritz; ZIE, Ziegenbuschhänge bei Oberau; WIP, Wipperdurchbruch; BOT, Bottendorfer Hügel; SBG, Schwellenburg; HOF, Hofberg; KOP, Koppelstein; DOE, Rheinhänge Dorscheider Heide.

fluopyram were found at 69 and 46%, respectively.

The ubiquitous presence of fungicides in soils observed in the present study – most notably of epoxiconazole, azoxystrobin, boscalid, and fluopyram, we believe is a result of their broad-spectrum application for control of fungal crop pathogens and their moderate to strong persistence in soils. Azoxystrobin alone has been approved for use on more than 80 crops (Herrero-Hernandez et al., 2015). Recent studies also detected fungicides frequently in EU agricultural soils (Silva et al., 2019; Manjarres-López et al., 2021). The most frequent CUP residues recorded in the present study in soils and vegetation are CUPs that have been widely used in different crops sometimes for over a decade. In some cases, frequent applications of pesticides can increase their (pseudo-) persistence in soil by continuously adding new residues to the soil. This can lead to the accumulation of the pesticide over time and potentially result in adverse effects on the environment. Furthermore, the most frequently detected CUP residues accounted for pesticide-active substances, which were sold at high volumes in Germany during the period 2008–2020.

For the CUP residues in soils reported in the present study, the highest total concentrations (sum concentrations >100 μg kg^{-1} are considered) were recorded at six (46%) of 13 sites, ranging from 141.5 to 1218.4 μg kg^{-1} (Fig. 4). The CUPs with the highest concentrations in single samples detected in the present study were pendimethalin (234.4 μg kg^{-1}, site: POR), azoxystrobin (303 μg kg^{-1}, MAL), and tebuconazole (514.6 μg kg^{-1}, POR).

For the CUP residues in vegetation, the highest total concentrations (>100 μg kg^{-1} are considered) were recorded at six (46%) of 13 sites ranging from 221.3 to 2661.6 μg kg^{-1} (Fig. 5). The CUPs with the highest concentration were spiroxamine (821 μg kg^{-1}, BOT) and epoxiconazole (2510 μg kg^{-1}, SBG). The most frequent CUPs, terbuthylazine (9 of 13 sites) and fluopyram (6 of 13 sites) exhibited concentrations of up to 14 (MAL) and 277 μg kg^{-1} (DOE), respectively.

The present data on the CUP residues show a tendency toward lower concentration levels in soils compared to that of vegetation. In this context, it is important to note that the total CUP concentrations in vegetation samples are dominated by one to three CUPs (Table S11). Elevated concentrations observed in the vegetation are likely due to a recent pesticide application that occurred prior to the sampling of both

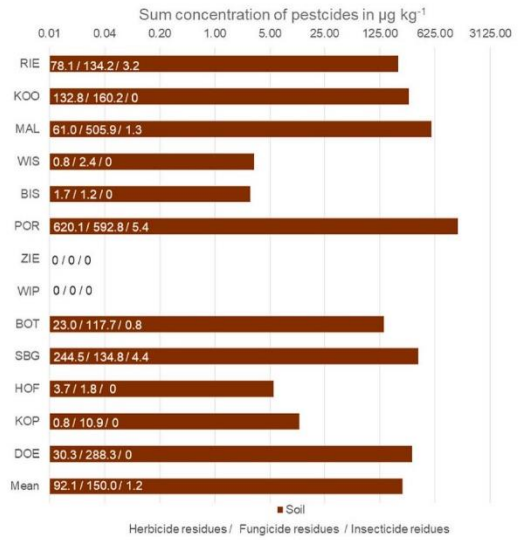


Fig. 4. Total CUP residue content in soil. Sum residue concentrations per pesticide class (= herbicides, fungicides, insecticides) and per sampling site are shown. For calculation of sum concentrations, only pesticide levels above the limit of quantification (LOQ) were considered.

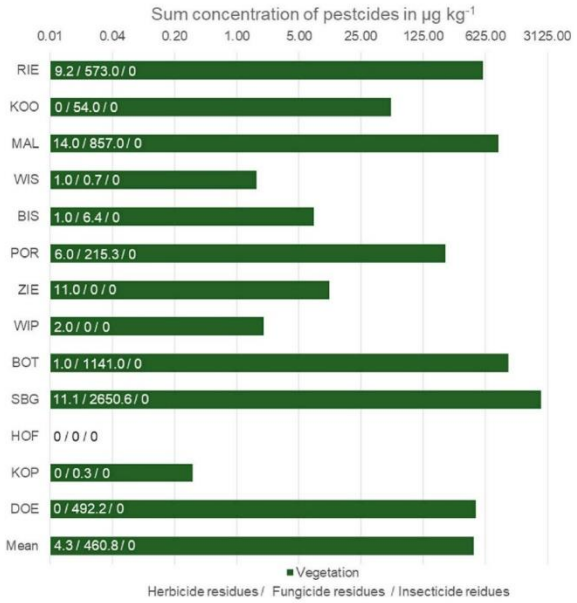


Fig. 5. Total CUP residue content in vegetation. Sum residue concentrations per pesticide class (= herbicides, fungicides, insecticides) and per sampling site are shown. For calculation of sum concentrations, only pesticide levels above the limit of quantification (LOQ) were considered.

the soil and vegetation. The crop stage at application may also explain the elevated concentrations in the vegetation. At the late crop stages, a larger fraction of the pesticide application rate is reaching the vegetation compared to the fraction reaching the soil. The interception fractions for arable crops (e.g. potatoes, rape, maize, cereals) at full growth may reach values of 0.8–0.9 compared to the soil deposit fractions of 0.1–0.2 (Linders et al., 2000), meaning that vegetation may receive up to 80–90% of the total application amount. The crop stage may also explain the presence of several CUPs detected only in soil. At the beginning of the vegetative period, at the early crop stage pesticides are primarily applied on the soil. Since the plants have not fully developed, their interception of the pesticides is low, resulting in higher deposit fractions in the soil.

It is possible that the CUPs found at lower concentrations in the vegetation may have entered through multiple pathways of pesticide exposure, such as input from the ambient air (Zaller et al., 2022) or uptake through the roots of the plants and translocation to above-ground parts (Hwang et al., 2017). It should be noted that in addition to the aforementioned pathways, such as uptake through roots and input from the ambient air, wet deposition during rain events could also be a contributing factor to the deposition of these CUPs in the sampled vegetation.

4. Conclusion

In the present study, a new sample preparation approach for the analysis of 98 current-use pesticides in soil and herbaceous vegetation using HPLC-MS/MS in combination with an acetonitrile-based extraction was developed and tested. The optimized and validated method was used for a CUP residue analysis of agricultural soils and crop vegetation from 13 agricultural sites in Germany. For both soil and vegetation, the recoveries (recovery, soil: 71–125%, vegetation: 70–117%) fulfiled the acceptance criteria for regulatory methods for all CUPs investigated. Our study's LOQs were lower for 27–100% of the shared CUPs depending on the previously published research. Our study revealed interesting

findings regarding the presence of CUPs in soil and vegetation. We observed that higher numbers of CUPs were detected at lower residue levels in soil, while the opposite was true for vegetation, where lower numbers of CUPs were found at higher residue levels. Additionally, we found that over 50% of the detected CUPs were classified as persistent to very persistent, based on their DT50 values. This suggests that a significant proportion of CUP residues in soil may originate from pesticide applications used in previous years, which could pose a threat to soil fauna and microbiome and have a detrimental effect on important soil processes such as nitrification and litter breakdown, ultimately degrading soil fertility over time. Therefore, our study highlights the need for systematic monitoring of CUP residues in agricultural soils and vegetation, and we have provided a validated HPLC-ESI-MS/MS method that can be used to facilitate this task for 98 commonly used pesticides.

Credit author statement

Nikita Bakanov: Conceptualization, Methodology, Validation, Investigation, Writing – Original Draft; **Carolina Honert**: Investigation, Writing – Review & Editing; **Lisa Eichler**: Software, Writing – Review & Editing; **Gerlind U.C. Lehmann**: Project administration, Writing – Review & Editing; **Ralf Schulz**: Writing – Review & Editing; **Carsten A. Brühl**: Supervision, Writing – Review & Editing.

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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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.138840.

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Appendix II: Paper 2

Assessment of current use pesticides in flower, pollen provision, and wild bees: HPLC-ESI-MS/MS method development and field implementation

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RESEARCH PAPER

Assessment of current use pesticides in flowers, pollen provision, and wild bees: HPLC-ESI-MS/MS method development and field implementation

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Abstract

Synthetic pesticide use is a major driver of pollinator declines in agricultural landscapes. To understand the impact of pesticides, it is essential to quantify residues in food resources and in insects themselves. We developed simple, fast, and cost-effective multiresidue methods for the simultaneous quantification of up to 83 current use pesticides (CUPs) in flowers (0.5 g sample weight) and 71 CUPs in pollen provision (0.1 g sample weight) via liquid chromatography-tandem mass spectrometry. Additionally, methods were developed for individual wild bees (*Osmia bicornis*), enabling the analysis of 65 CUPs in 0.02 g samples (females) and 45 CUPs in 0.01 g samples (males). The extractions used acidified acetonitrile (2.5% formic acid), with phase separation assisted by ammonium formate and clean-up via freeze-out. The validation showed limits of quantification between 0.00025 mg/kg and 0.05 mg/kg for flowers, 0.0002 mg/kg to 0.052 mg/kg for pollen provision, 0.0002 mg/kg to 0.08 mg/kg for female bees, and 0.00008 mg/kg to 0.1 mg/kg for male bees. The methods were applied to flowers, pollen provision, and post-pupal bees from agricultural sites. In total, 47 CUPs were detected in flowers, 35 in pollen provision, and 4 in post-pupal bees, with herbicides being most prevalent. This study highlights the exposure of pollinators to CUP mixtures, including emerging bees that have not yet been active in the environment. Our methods provide practical tools for monitoring CUP residues in small environmental samples, supporting the assessment of exposure in plant-insect matrices.

Keywords Pesticides · Agriculture · Insects · Contamination monitoring · HPLC-ESI-MS/MS

Introduction

The widespread use of synthetic pesticides for pest control has resulted in their ubiquitous presence in various parts of the biosphere [1, 2]. Current use pesticides (CUPs) have been detected in soil and vegetation at large scales, creating chemical landscapes and showing that CUPs are distributed further from the cropping area, as assumed by drift estimations [3, 4]. Exposure to pesticide mixtures has been suggested to be a major factor in the decline in pollinating insects [5, 6].

Recently, studies on insect exposure to pesticides in terrestrial ecosystems have increased, revealing multiple CUP residues in pollen from flowers [7, 8] or collected by honey bees (*Apis mellifera*, bee bread) [9–13] and bumble bees [8, 12]. CUP mixtures have also been recorded in [14–17] and on insects [18]. These findings emphasise the need for monitoring approaches to understand the presence of pesticide mixtures in terrestrial ecosystems and adjust current risk assessment practices [19].

Despite the direct contact of insects with flowers, research on CUP residues in blossoms remains limited. Existing approaches for the extraction of CUPs from flowers typically adapt protocols from food analysis (e.g. [20–22]). Sampling insects, flowers, or pollen is further constrained by the limited availability of matrix material in natural environments. Common extraction methods are based on the QuEChERS approach [23] and involve the use of a larger amount of sample material, usually 10 g for vegetables, fruits, and soil [24]. For pollen analysis, typically quantities between 1 and 5 g [25] are used. This is easily achievable in the context of honey bees, due to the storage of large quantities of pollen as bee bread. Obtaining sufficient wild bee pollen necessitates pooling larval pollen provision from multiple brood

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cells, obscuring individual exposure data. The development of small-scale extraction methods is essential to prevent the loss of information in combined samples, to evaluate the exposure of individuals separately [7], and to enable monitoring approaches.

We studied the red mason bee *Osmia bicornis*, which is also used as a surrogate test species in the registration process of pesticides [26]. The females of this species construct brood chambers in tubular passages in dead wood, with individual brood cells separated from each other by soil material. Each brood cell contains a supply of pollen, with the quantity stored varying depending on the sex of the developing larva. The pollen quantity ranges from 0.075 to 0.525 g of pollen, with 0.15 g being common [27]. Owing to the initial consumption of the pollen provision provided for the larvae, 0.1 g was chosen as a realistic sample amount for CUP analysis. With current approaches, it is not possible to analyse this low volume of pollen collected for the provision of single wild bee larvae.

For insects, in most published methods, multiple individuals are combined to obtain a sufficient sample size. Residues of CUP were recorded in honey bees *Apis mellifera* [10], as well as in wild bees [15], where multiple individuals were used to obtain between 0.083 g to 1.9 g [15] and 5 g of bees [10] for extractions. An exception is a British study, where CUPs from single bumble bees *Bombus terrestris* with an average weight of 0.098 ± 0.030 g were measured [7]. We aimed to determine the CUP residues in a single bee individual to compare them with the residues of stored pollen provision. Moreover, a small-scale method is more appropriate for monitoring approaches, as it results in less interference with the ecosystem. To the best of our knowledge, no methods for isolating single small solitary wild bees have been published thus far. Therefore, we had to develop a method that could be used for a single individual. The dry weight of a female *Osmia bicornis* varies between 0.022 g and 0.04 g, and for a male, it varies between 0.012 g and 0.024 g; therefore, 0.02 g and 0.01 g were selected to ensure the measurability of all the sampled individual females and males, respectively.

The central aim of this study was to develop efficient methods for the extraction and detection of multiple CUPs from low-mass samples of flowers (0.5 g), larval pollen provision (0.1 g), and newly hatched wild bee individuals (0.02 g and 0.01 g). The simplified extraction approach with minimal steps and components to reduce processing time, minimise the probability of human error, and, in particular, allows a cost-efficient processing of many environmental samples in a short time. The methods were validated and applied to field-collected samples of flowers, pollen provision, and newly hatched *Osmia bicornis*. Furthermore, we aimed to find a contamination path throughout the matrices by detecting the same pesticides in all the matrices.

Material and methods

Analytical standards, chemicals, and reagents

For the pesticide stock solutions, 93 CUP reference standards (purity ≥ 98%) were purchased either from Restek (Bellefonte, PA, USA) as multicomponent solutions (concentration, 100 mg/L) or from Dr. Ehrenstorfer (Augsburg, Germany) and Sigma-Aldrich (St. Louis, MO, USA) as single-component standards or solutions. A list of all standards is provided in Electronic Supplementary Material (ESM) Table S1. Single-stock and multicomponent solutions were combined into the final multicomponent solution (containing each analyte at a concentration of 10 mg/l) immediately before use to prevent the degradation of sensitive analytes. The final multicomponent solution was further diluted with HPLC-grade acetonitrile (≥99%, Honeywell, Seelze, Germany) to prepare five working solutions, each containing all the analytes at concentrations of 0.0005, 0.001, 0.01, 0.1, and 1 mg/l. All the solutions were stored at −20 °C in a freezer. For preparation of the HPLC mobile phases, LC-MS grade methanol (≥99.9%, Honeywell, Seelze, Germany), water (Merck, Darmstadt, Germany), buffer/additive, formic acid (≥99%, VWR International, Leuven, Belgium), and ammonium formate (NH_4HCO_2, ≥99%, Sigma-Aldrich, St Louis, MO, USA) were used. For the extraction, ammonium formate and HPLC-grade acetonitrile were used. Deuterium-labelled imidacloprid (purity ≥ 98.9%, Dr. Ehrenstorfer GmbH, Augsburg, Germany) was added to each sample prior to extraction as part of the quality assurance. For the clean-up steps, C18 (SPE Bulk Sorbent, Agilent Technologies, Santa Clara CA, USA), Z-Sep+ (Supel™ QuE Z-Sep+, Supelco®, Bellefonte, USA), and PSA (SPE Bulk Sorbent, Agilent Technologies, Santa Clara CA, USA) were used.

Analysed pesticides

The 93 analysed current use pesticides (CUPs) were selected on the basis of records from the Julius-Kühn Institute (JKI) published in the PAPA database (JKI, 2019), as well as JKI records for the frequency of pesticides in 2016 and 2017. The most commonly used herbicides, fungicides, and insecticides for winter wheat, oilseed rape, maize, potato, and grapes were chosen. Additional information on the selected CUPs is available in a previous publication [28]. In contrast to the previously selected CUPs [28], avermectin (biocide) has been replaced by dimethoate (insecticide). The aim was to adapt the methods for flowers, pollen, and individual wild bees. The existing methods involve 93 target analytes, including 36

fungicides (F), 36 herbicides (H), and 21 insecticides (I). In this study, we report the determination of pure biologically active isomers, namely, dimethenamid-P (H), metolachlor-S (H), metalaxyl-M (F), spinosad-A (I), spinosad-D (I), and quizalofop-P (H), as the sum of isomers because commercial pesticide formulations often contain mixtures of active and nonactive stereoisomers.

HPLC-ESI/MS-MS analysis

Analyte concentrations were measured via high-performance liquid chromatography coupled with tandem mass spectrometry via electrospray ionisation (HPLC-ESI-MS/MS; LC: Agilent Technologies LC 1260 Infinity II series, MS/MS: Agilent Technologies 6495 C, Santa Clara CA, USA). The mass spectrometer was operated in multiple reaction monitoring (MRM) mode with two characteristic MRM transitions per analyte (except for proquinazid, where only one MRM transition was used, ESM Table S2). Analytes were quantified via matrix-matched external calibration standards. For the calibration standards, blank matrix samples were fortified with working solutions to achieve final analyte concentrations of 0.025, 0.05, 0.1, 0.5, 1, 10, 50, and 100 µg/l. Calibration standards were injected before and after each sample batch or after every 20 samples to monitor for changes in the sensitivity of the mass spectrometer due to residues of the sample matrix in the ion source. Additionally, an internal standard was added to all the environmental samples prior to extraction to monitor for variation in the extraction procedure.

An Agilent MassHunter Workstation (Quant-my-way, Quantitative analysis for QQQ version 10, Agilent Technologies, Inc., Santa Clara CA, USA) was used to process the generated chromatograms. Analytes in the samples were identified by comparing the peak retention time and qualifier/quantifier MRM (ESM Table S2) ratios to those of the calibration standard at a similar concentration and comparing the values to the limits of detection (LODs, ESM Table S3) and quantification (LOQs, ESM Table S3) determined during method validation. The tolerance based on the qualifier/quantifier MRM ratios was defined to be within ± 20%.

Samples and sample treatment

Materials used for method development

To prepare matrix-matched calibration series and perform recovery experiments of fortified samples, an uncontaminated reference material was sourced for each matrix. For this purpose, we collected flowers from the surroundings (parks, urban areas) of the Institute for Environmental Sciences Landau, RPTU, Germany (49°12′13.7″N 8°06′20.2″E). These flowers served as blank material but still contained residues of four CUPs (fluopyram, prosulfocarb, terbuthylazine and trifloxystobin). For the pollen method, commercially available pollen products, all of which contained CUPs, were tested. Alpine pollen (Blütenpollen Alpenmischung, GRAZE der Imkershop, Weinstadt, Germany) was chosen as the blank material because it contained the fewest CUPs (10 analytes). Adult red mason bees (*Osmia bicornis*, purchased from WAB-Mauerbienenzucht, Konstanz, Germany), which hatched in the laboratory, were used as blank material for the bee method. For the flower and pollen analyses, the concentrations of the CUP residue in the calibration series were adjusted by subtracting those detected in the blank material. The flowers and bees were freeze-dried (Alpha 1–4 L5 Cbasic, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) to ensure a homogeneous mixture. The drying process was not necessary for pollen provision, as pollen is a dry pellet that is easily homogenised during milling. All blank materials were stored in Eppendorf tubes at −20 °C prior to method development. The samples were milled to powder (pollen and flowers with an IKA, TubeMill control 100; IKA Staufen, Germany; bees with an oscillating mill, Typ MM 301, Retsch GmbH, Haan, Germany; 3 × 1 min; 30 × 1/s).

Environmental samples

All field samples were collected as part of the InsectExpo project [2]. The samples were collected at the same sites described in a previous work [2]. Nine agricultural sites (arable land (n=3), vegetable cultivation (n=3), and viticulture (n=3)) and adjacent meadows in the Rhineland-Palatinate region of southwestern Germany were investigated. Samples were taken in March and June 2021. Flower samples for this study were taken from meadows bordering the fields in the main wind direction to the east at distances of 1 m. After the flower species were identified, they were collected in separate sample bags (Rotilabo® sample bags, LDPE, Carl Roth GmbH + Co. KG, Karlsruhe, Germany). In the laboratory, all the samples were directly frozen (−20 °C). If enough flower material was available (more than 6 g dry weight), the flowers of an individual species were analysed. In several cases, flower mixtures had to be prepared to obtain the required amount of material (0.5 g), as the number of flower sources was limited. For mixtures, samples from the same date and sample site were pooled.

Nesting aids [29] and *Osmia bicornis* cocoons were placed in all the meadows (n=9) from the end of March to the end of July 2021. Initially, 13 female cocoons and 7 male cocoons were placed in paper rolls beneath the nesting aids at each sample site. After 2 weeks, 10 female and 10 male cocoons were added every 2 weeks to obtain constantly active bees, and pollen was continuously collected by the

bees to provide pollen provision for the larvae. Nesting aids were built from three nesting boards of milled MDF boards (16 × 16 × 1.6 cm) with 10 nesting tunnels (8 mm, www.mauerbienen-shop.de, WAB-Mauerbienenzucht, Konstanz, Germany). To protect brood cells during handling, acetate sheets were fixed to each nesting board with adhesive strips. A 12-mm mesh net shielded the bees from predators, and nesting aids were secured with lashing straps on wooden posts, at least 1 m above the ground and 20 m from agricultural land, oriented south/southeast. The development of the nesting aids was regularly photographed to document brood cell establishment during the study. When a nesting board was full of brood cells or after 3 weeks, the nesting boards were taken to the laboratory before all the pollen was consumed by the larvae. Pollen samples were carefully taken from single brood cells via a spatula, and the larvae were subsequently fed purchased pollen but were not used for this study. Larval development from neighbouring cells, built within ± 1 day and at the same location, was observed, and after diapause (October to March, in a refrigerator, 3 °C), hatching success was documented. The newly hatched bees were directly frozen (−20°C). Bees that did not hatch after 4 weeks from the end of hibernation (14.03.22, first hatching bee: 20.03.22, last hatching bee: 08.04.22) were manually removed from the cocoon and frozen. All post-pupal bees (n=33) were later used for pesticide residue analyses.

Development of the extraction procedure

For all the matrices, different extraction steps were investigated to achieve simple, quick, effective, and inexpensive extraction of multiple CUPs. The solvent extractions were based on extraction methods previously developed in our laboratory for soil and vegetation [28] and for biota [30]. For the experiments, a precise mass (Sartorius CP225D, Göttingen, Germany) of the sample was fortified with a standard solution of CUPs prepared in acetonitrile and evaporated to dryness under a fume hood (for at least 30 min). All shaking steps were performed via an end-over-end overhead rotator (Stuart STR4/2, Keison Products, Chelmsford, UK). For centrifugation, a MegaStar 1.6R (VWR, Radnor, USA) was used. Method average recoveries (recovery, REC %) and precision (relative standard deviation, RSD %) were evaluated by comparing fortified blank samples before extraction (n=3) and post-extraction fortified sample (n=1) for each matrix individually.

Flowers

For the pesticide extraction of flower material, 0.5 g dry weight (dw) of flower powder was weighed into a 50-ml CellStar® tube (PP conical, Greiner Bio-One, Frickenhausen, Germany). For each experiment, three samples were fortified with the working solution, while an additional three samples were left as blanks for spiking after the extraction process. For extraction, 10 ml of acetonitrile containing 2.5% formic acid (FA) and 0.25 g of ammonium formate was added to the fortified and blank flower samples. The samples were agitated for 1 h via an end-over-end overhead rotator and centrifuged (6 min, 3000 rpm). The supernatant was decanted and filtered through 0.2-µm filters (17-mm HPLC syringe filter, PTFE, hydrophobic, BGB Analytik, Lörrach, Germany) into 20-ml glass vials.

To eliminate potentially interfering substances, such as pigments or oils, we evaluated three dispersive solid-phase extraction (dSPE) clean-ups using either (1) PSA or (2) C18 as a sorbent or (3) freeze-out with cooled filtration. For dSPE, 1 ml of the supernatant was added to an Eppendorf tube containing 50 mg of sorbent, and the samples were then vortexed for 30 s and filtered into an HPLC vial. For freeze-out, the samples were subjected to freeze-out overnight at −20 °C (12 h), then placed on ice and filtered through PTFE filters (17 mm HPLC syringe filter, PTFE, pore size 0.2 µm; La-Pha-Pack, Langerwehe, Germany) into HPLC vials, ensuring that they remained cold during the filtration step. For the final method, 0.5 g dry weight of flowers sampled in the field was extracted with 10 ml of acetonitrile containing 2.5% FA and 0.25 g of ammonium formate, and a freeze-out was used as a clean-up.

Pollen provision

We used 0.1 g of pollen provision, as this was a realistic expected quantity per brood cell [27]. For the extraction of the pollen provision, 0.1 g fresh weight (fw) of pollen was weighed into 15-ml cellstar® tubes (PP conical, Greiner Bio-One, Frickenhausen, Germany). To remove proteins and fatty acids from the pollen matrix, different purification methods were tested. The supernatant was either (1) frozen overnight (freeze-out) followed by cooled filtration or cleaned by dSPE with (2) C18, (3) PSA, or (4) Z-Sep+. For the clean-up with PSA, 1 ml of H_2O was added before agitation [31]. After solvent extraction, 1 ml of the supernatant was placed in a tube containing (1) 100 mg of C18, (2) 150 mg of MgSO4 + 25 mg of PSA, or (3) 100 mg of Z-Sep and vortexed for 30 s. The samples were centrifuged (6 min, 3000 rpm), and the supernatant was filtered on ice through 0.2-µm filters into an HPLC vial. For freeze-out, the supernatants of the samples were stored in a freezer (−20 °C, 12 h), after which the freeze-out samples were filtered before analysis. For the measurement of the field samples, the final method included the use of 2.5 ml of acetonitrile, 2.5% FA, 0.1 g of ammonium formate, and a freeze-out.

Post-pupal bees—*Osmia bicornis*

For individual red mason bee (*Osmia bicornis*) extraction, we tested an ultrasonic bath extraction method followed by purification via PSA, and Z-Sep and nitrogen evaporation. The method was partially based on the AOAC QuEChERS method [32] and a method that was previously established for small sample sizes of insects [30]. For extraction, 300 µl of water was added to 0.020 g of dried female bee powder in 2.5-ml Eppendorf tubes, which were subsequently vortexed for 30 s. Then, 2000 µl of 1% acetonitrile was added, and the mixture was vortexed again. Salts (10 mg of NaOAc, 40 mg of MgSO4, and 10 mg of NACl) were added, and the mixture was vortexed, followed by a 5-min ultrasonic bath (Sonorex Digitec, Bandelin, Berlin, Germany) and a centrifugation step (5 min, 4700 rpm). A total of 1800 µl of the supernatant was added to a vial containing Z-Sep+ (SupelTM QuE QuEChERS, Merck KGaA, Darmstadt, Germany) (0.0008 g per mg of bee powder) and then to a vial containing PSA (0.0008 g per mg of bee powder). The mixture was vortexed for 30 s and then centrifuged (5 min, 4700 rpm) again. The supernatant was evaporated to dryness under a nitrogen stream, and the resulting extract was dissolved in 500 µl of a solution containing water-methanol (H_2O/MeOH, 70:30) and 0.1% FA. The solution was then filtered through PTFE filters (0.2 µm, 4-mm syringe filter, OTFE, Resteck, Bad Homburg, Germany).

Additionally, we tested a simple liquid extraction method, which has already been established for 93 CUPs for other environmental samples [28], with different clean-up steps ((1) C18, (2) PSA, (3) freeze-out + cooled filtration). For this method, 0.5 ml of acetonitrile, 2.5% FA, and 0.020 g of ammonium formate were added to 0.020 g of bee powder in a 2.5-ml Eppendorf tube, followed by 1 h of agitation, a centrifugation step (6 min, 3000 rpm) and the transfer of the supernatant by filtering into another Eppendorf tube. For three different clean-up steps, the supernatant was added to (1) C18 (0.0008 g per mg bee powder), (2) Z-Sep + PSA (0.0008 g per mg bee powder), or (3) freeze-out (−20 °C, 12 h) + cooled filtration. The samples were subsequently vortexed, centrifuged (5 min, 4700 rpm) and filtered (0.2 µm, 4 mm). For (3), the samples were centrifuged under cooled conditions (−10 °C).

For the post-pupal bees that hatched in the laboratory or were manually removed from the cocoons after consuming the provided pollen provision collected in the field, we used the final method of 0.5 ml of acetonitrile containing 2.5% FA, 0.02 g of ammonium formate, and a freeze-out as a clean-up for 0.02 g of dried females. The method was also validated for 0.01 g of dried males with 0.5 ml acetonitrile 2.5% FA and 0.01 g of ammonium formate; after agitation and centrifugation, samples with 0.01 g matrix were evaporated for 30 min using nitrogen. The samples were resolved in 0.25 ml acetonitrile 2.5% FA and further treated like the 0.02 g samples.

Method validation

The optimised extraction methods were validated for the analysis of 93 CUPs in flowers, pollen, and female and male bees by analysing accuracy (recovery, REC (%)), precision (relative standard deviation, RSD (%)), linearity (R^2), matrix effects (ME (%)), and limits of detection and quantification (LODs and LOQs). If one of the validation parameters (REC, RSD, or ME) was not achieved, the substance failed to be validated.

RECs were determined by calculating the ratio of the response of blank samples fortified with two concentrations prior to extraction to the response of the blank samples fortified after extraction with a minimum of three repetitions. The REC needed to be in the range of 70–120% [33]. The RSDs of the methods were evaluated by determining the relative standard deviations (RSD=(100*standard deviation)/average) of at least three repeated extractions of each matrix spiked at two concentrations (for flowers, pollen provision, and female bees (20 mg): 0.01 mg/kg and 0.1 mg/kg dry weigh; for male bees (10 mg): 0.1 mg/kg and 0.25 mg/kg). Acceptable precision was achieved if the RSD was <20% [33]. R^2 was evaluated via matrix-matched calibration curves prepared as described above and in a concentration range of 0.00025–2 mg/kg (8 concentrations). Linearity was considered acceptable if R^2>0.99. The ME was evaluated by comparisons of the slopes of the calibration curves (eight concentrations) in solvent only (acetonitrile, 100%) and in the matrix. The percent increase or decrease in the slope of the matrix-matched calibration curve was measured in relation to the solvent-only curve. MEs less than 20% were considered acceptable [33]. The LODs and LOQs were determined from spike samples (n=5 for each of the eight calibration levels). The LOD (LOD = (3* standard derivation of the response)/slope of the calibration curve) was determined as the minimum amount of analyte for the quantifier multiple reaction monitoring (MRM) transition detected with a signal-to-noise (S/N) ratio of three, and the LOQ (LOQ= (10*standard derivation of the response)/slope of the calibration curve) was determined as the minimum amount of analyte detected at an S/N ratio of ten. For the determination of the LOD and LOQ, five blank samples per concentration were fortified after extraction. The signal-to-noise ratio was calculated with a root mean square (RMS) algorithm in the MassHunter Workstation.

Statistical analysis

For significant differences between the methods, an ANOVA test (package: stats [34]) and a post hoc t-test were

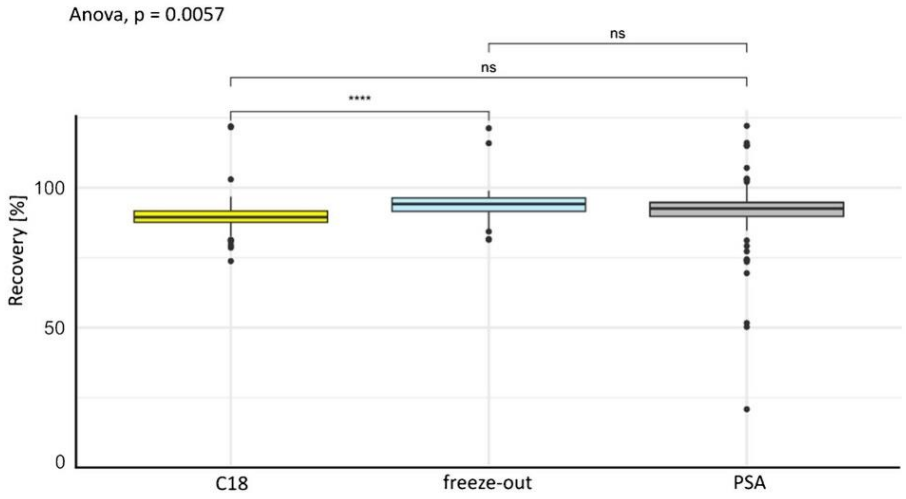


Fig. 1 Boxplot of recoveries [%] over all substances (n=93 CUPs) with the tested methods (n=3, dSPE clean-up with C18 (yellow) and PSA (grey), as well as a freeze-out (light blue) as a clean-up) for 0.5 g of flower material. Lines indicate significance (****) or non-significance (ns) (t test, CL= 95%)

conducted [35]. Plots were produced with ggplot (package: ggplot2 [36]). For the environmental samples whose detection results were below the limit of quantification but above the limit of detection, the detections were not used for cumulative mixture CUP concentrations but were counted for the number of CUPs. Owing to nonnormally distributed data, we used the Kruskal-Wallis test (package: stats [34]) and Dunn post hoc test (package: FSA [37]) for multiple comparisons between types of agricultural sites (arable sites, vegetable sites, viticulture sites) per matrix and between sample types (flowers, pollen provision, post-pupal bees). For cumulative concentrations, the data were log transformed. All the statistical analyses were performed via R software (version 2023.06.2+561, R Core Team, 2022).

Results and discussion

Method development and validation

To detect multiple CUPs in environmental samples such as flowers, pollen provision, and wild bees, multiresidue methods capable of analysing a broad range of analytes have been developed. To ensure appropriate extraction conditions for acidic and basic compounds, acetonitrile with formic acid and ammonium formate were used as buffers [38]. The aim was to develop effective and inexpensive methods that enable a large number of environmental samples to be analysed quickly.

Flowers

For the flower extraction method, different clean-up methods were tested: overnight freeze-out and dSPE with C18 and PSA (see Fig. 1). The average REC after dSPE with C18 (REC: 90 ± 6%) was statistically significantly lower than that after the freeze-out method (freeze-out: 94 ± 5%; see Fig. 1). No statistically significant differences were found between the PSA method (PSA: 91 ± 12%) and the freeze-out or the PSA and C18 methods. PSA and C18 are commonly used for clean-up steps. PSA is capable of retaining fatty acids from acetonitrile [39]. C18, on the other hand, is characterised by non-polar properties and is able to adsorb fatty acids due to hydrophobic interactions. Due to the low solubility of lipids and proteins, these compounds precipitate on the tube surface during a freeze-out process. As a result, the supernatant can be transferred without these compounds. Although PSA showed comparable recoveries, freeze-out was universally favoured due to its simplicity, lower cost, and lower methodological complexity and as the analytical sensitivity was sufficient for multiple target analytes and an additional clean-up step would have significantly reduced throughput. Owing to the simplicity of the method, overnight freeze-out was chosen over PSA.

Validation Among the 93 CUPs, 83 achieved the aforementioned validation criteria. The RSDs were within the acceptable range for 88 and 92 of the 93 tested CUPs, depending on the fortification level. Depending on the fortification levels, RSD ranged from 1.3% (hexythiazox, I) to 12.7% (difenconazole, F) for validated CUPs in the flower samples, with 98% (n=81 of 83 validated CUPs) of the CUPs having RSDs smaller than 10%. Acceptable REC were achieved for 87 and 91 of the 93 CUPs depending on the fortification level, with average recoveries ranging between 76.3% (propamocarb, F) and 118.9% (flonicamid, I). The R^2 was ≥ 0.99 for 92 of the 93 tested CUPs. For the validation of the CUPs in flowers, 90 CUPs had MEs (between −20 (clomazone, I) and 13% (benalaxyl, f)) considered negligible (ESM Table S3).

The LOQ for flowers varied from 0.00025 mg/kg (propaquizafop, H) to 0.02 mg/kg (aminopyralid, H) (ESM Table S3). Among the 83 CUPs, 20 had LOQs ranging from 0.00025 mg/kg to 0.001 mg/kg, 49 had LOQs between 0.0011 mg/kg and 0.01 mg/kg, and 5 had LOQs between 0.010 mg/kg and 0.1 mg/kg (ESM Table S3). To further improve the LOQs, additional experiments with different extraction steps could be conducted. For example, evaporating the solvent and reabsorbing it into another solvent could improve recovery.

The detection limits of our method are lower than those of other studies investigating CUP residues in flowers (LOD: 0.006 mg/kg for LC analytes and 0.016 mg/kg for GC analytes, LOQs not mentioned [15] and LOQs: <0.01 mg/kg, LODs not mentioned [20]). In contrast to our method, the methods used in these studies were not initially developed and validated for flowers. Low LOQ values are particularly important when analysing environmental samples, as CUPs are often present at low concentration levels and in complex mixtures (e.g. (2), see also the "Method application" section). Even trace-level CUP residues on flowers can lead to contact exposure for flower-visiting insects. Although the effect of such low concentration exposure has not yet been studied, this exposure pathway should be further investigated [6]. For example, acetamiprid, a common insecticide, has a contact lethal dose (LD50) of 9.26 µg/honey bee [40]. While it seems unlikely that this dose is reached through contact with flowers, it is important to note that acetamiprid present in mixtures can exhibit synergistic effects on honey bees [41]. Moreover, lethality threshold concentrations provide only a rough estimate of toxicity. The distinction between lethal and sublethal effects is often difficult and depends on various factors, including individual fitness or population health and environmental factors [42]. In three Belgian studies, 10 g of cut flowers was analysed to identify approximately 500 target CUPs [20, 22]. The samples were analysed by a laboratory specialising in residue analysis for food and herbs, following a QuEChERS approach [43] involving the use of 10 ml acetonitrile with 1% acetic acid and QuEChERS salts (4 g anhydrous magnesium sulfate, 1 g sodium acetate), shaking and a sonication step in an ultrasonic bath [22], and the use of clean-up tubes [20, 22]. The extracts were measured both by liquid chromatography (LC) and gas chromatography (GC). This enables the detection of up to 500 CUPs. However, the use of magnesium sulfate leads to an exothermic reaction, and this increase in temperature worsens the detection of more unstable pesticides [44]. Moreover, ultrasonic baths have also been used to remove CUPs from eatables [45]. Ultrasonic sonification also occurs at relatively high temperatures (e.g. between 26 and 36 °C [46]), leading to loess with temperature-unstable CUPs. Comparisons of the recovery of CUPs extracted with ultrasonic bath or milder conditions due to overhead shaking obtained better results for the selected CUPs [28]. Furthermore, a sample amount of 10 g is achievable in the context of commercial flowers but not in natural habitats. In this context, floral pollen samples were analysed; however, flower-visiting insects are exposed also via the flower surface, which serves as a contact exposure matrix for these insects. This is particularly of concern regarding CUPs with high lipophilicity, as these substances might permeate the cuticular lipid layers of insects during foraging or nesting [47].

In order to determine the exposure of wild bees and honey bees to CUPs from flowers from field margins of agricultural land, in a Californian study, flowers were extracted by a pressurised liquid extraction followed by a solid-phase extraction clean-up and analysed for 163 CUPs [17]. Here, a 0.3-g sample was used, which is similar to our method (0.5 g of flower). However, the extraction involves temperatures up to 100 °C [15], causing possible losses, as discussed above. The samples were again measured by both LC and GC, which cover a broader range of CUPs. An additional measurement of our extracts with GC-MS/MS would be a possibility to cover more CUPs. Owing to the absence of water in our extracts, an adaptation of the method for additional measurements with GC is imaginable.

Pollen provision of bee larvae

Common clean-up approaches for pollen samples involve manual dSPE with C18, PSA (e.g. [11]), or Z-Sep as sorbents (e.g. [48]). Z-Sep is a mixture of C18 and zirconium dioxide sorbents and is used to reduce fats and proteins; it was successfully used in other studies investigating extraction methods for pollen [49]. However, in our experiments, the REC achieved via Z-Sep was generally below the desired range for most of the analytes. This outcome (mean REC: 64 ± 10% for all 93 CUPs) suggests that the quantity of Z-Sep might have been excessive and that further optimisation with reduced amounts could yield more recoveries within the range. In contrast, the recoveries for most of the analytes fell within the target range of 70–120% when clean-up methods with C18 (mean REC: 95 ± 11% for all 93 CUPs), PSA (mean REC: 99 ± 34% for all 93 CUPs), or freeze-out (mean REC: 94 ± 11% for all 93 CUPs) were used. No statistically significant differences were detected between PSA, C18, and freeze-out (see Fig. 2, ANOVA, p value = $2.2e^{-16}$, ESM Table S3). The method with overnight freeze-out was chosen because of its lower cost and fewer steps, as it requires no sorbent and is thus simpler, reducing the likelihood of human error. However, the use of further clean-up reagents might decrease MEs for some substances, as has been shown in other studies (e.g. [49, 50]).

In another method, 0.1 g of pollen material collected directly from flowers was used, as the authors aimed to

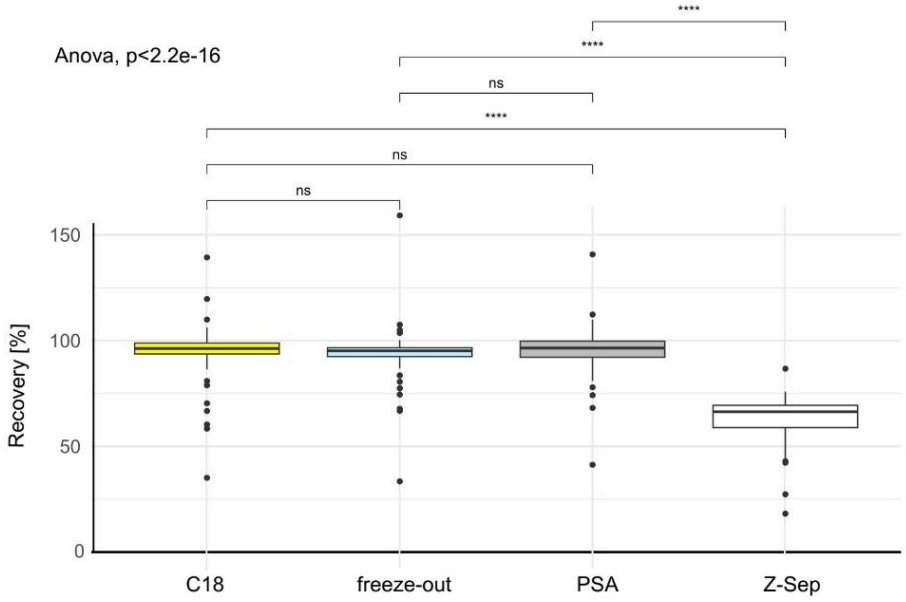


Fig. 2 Boxplot of recoveries [%] over all substances (n=93 CUPs) with the tested methods (n=4, dSPE clean-up with C18 (yellow), PSA (grey), and Z-Sep (white), as well as a freeze-out (light blue) as a clean-up) for 0.1 g pollen provision. Lines indicate significance (****) or non-significance (ns) (t test, CL= 95%)

analyse the exposure of pollen from individual plant species [7]. This method would have also been suitable for our sample amount of 0.1 g. However, the extraction process involves several steps, including dSPE, evaporation under vacuum, and reconstitution, increasing the time and complexity of the procedure.

Freeze-out was previously used in other pollen or bee bread extraction methods. For the analyses of 267 different CUPs in bee bread, a freeze-out alone did not yield satisfying results; therefore, two dSPE steps were included [38]. Other authors reported that an additional dSPE with PSA+C18 followed by an SPE step with Z-Sep after freeze-out was most efficient for the extraction of 220 CUPs with LC and GC [49].

Validation The method was validated successfully for 71 of the 93 tested CUPs (ESM Table S3). RSDs were in acceptable ranges for 83 and 90 of the 93 tested CUPs depending on the fortification level, ranging between 2.5% (picoxystrobin, F) and 17.9% (boscalid, F). Acceptable recoveries were achieved for 87 and 93 of the 93 tested CUPs again depending on the fortification level, with REC between 88.2% (myclobutanil, F) and 119.4% (cymoxanil, F). Linearity showed acceptable coefficients ($R^2 \geq 0.99$) for 92 of the tested CUPs. The MEs were negligible for 80 CUPs; MEs for validated CUPs (n=71) ranged from −18.8% (acetamiprid, I) to +17.0% (florasulam, H).

The LOQs ranged from 0.0002 mg/kg (propamocarb, F) to 0.052 mg/kg (bromoxynil, H) (ESM Table S3). Among all the analysed CUPs in pollen provision, 20 had LOQs lower than 0.001 mg/kg, 46 had LOQs between 0.0011 mg/kg and 0.01 mg/kg, and 5 had LOQs between 0.011 mg/kg and 0.1 mg/kg. A recent study has shown that environmental low concentration of CUPs in mixtures leads to sublethal effects on insects [51]; for this reason, it was desired to establish low LOQ values. The overnight freeze-out method was finally validated for 71 CUPs.

Post-pupal bees

For post-pupal bees, we compared the method that we used for pollen provision extraction to solid-liquid extraction via acidified acetonitrile followed by dSPE clean-up via Z-Sep and PSA, which has been applied to extract multiple analytes from small arthropod samples by other authors ("biota" method) [30].

The REC was statistically significantly lower (ANOVA, $p < 0.05$) across all the tested CUPs for the dSPE with C18 (mean REC: 57 ± 20%) and PSA with C18 (mean REC: 50 ± 26%) than for the freeze-out samples (mean REC: 92 ± 23%). The mean RECs for the "biota" method were 94 ± 94%, which was significantly different (ANOVA, $p < 0.05$; ESM Table S8) from those of the methods including dSPE (see Fig. 3). We opted for the pollen provision-based method with a freeze-out of our environmental samples, as RECs were within the range for more CUPs and the simplicity of this method, as discussed previously for the other matrices.

Validation The methods were successfully validated for 65 (female bees, 20 mg dw) and 45 (male bees, 10 mg dw) CUPs. Validation for female bees failed due to poor REC values (n=21, ESM Table S3), poor RSD (n=16), or poor ME (n=5). For the male matrix, the most common unfulfilled

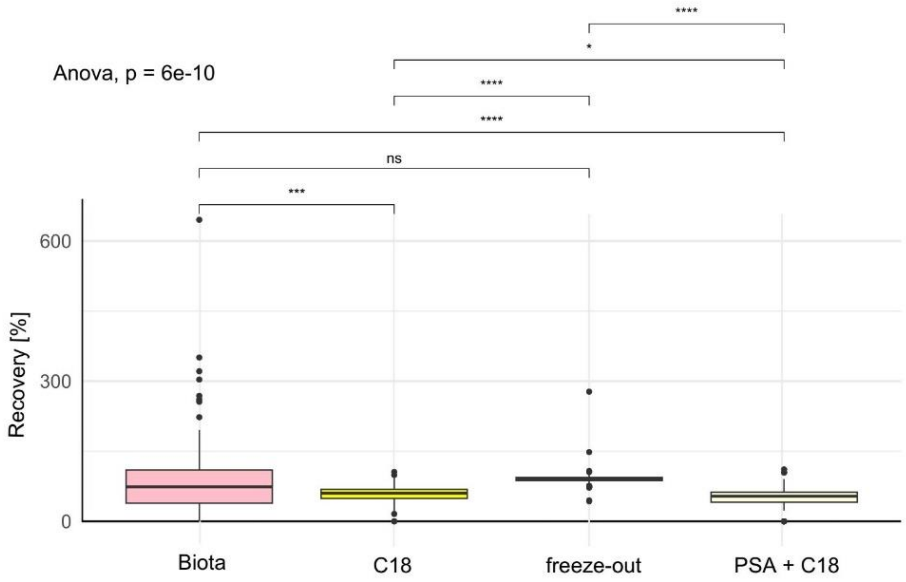


Fig. 3 Boxplot of recoveries [%] over all substances (n=93 CUPs) with the tested methods (n=4: the "Biota" extraction method (red) based on an extraction method for biota [52], extractions based on the proposed pollen provision extraction and a dSPE clean-up with C18 (yellow) and PSA+C18 (eggshell) as well as a freeze-out (light blue) as a clean-up) for 0.02 g bee powder. Lines indicate significance (*, ***, ****) or non-significance (ns) (t test, CL= 95%)

criterion was the ME (n=41, ESM Table S3). In contrast to the other matrixes, extracts of male bees were evaporated to dryness before resolving in acetonitrile, whereby unremoved fats or other co-extracts can be concentrated, which might have led to higher ME. Additional steps with hexane could be tried out in further investigations, as a fraction of hexane in acetonitrile is known to remove lipids from extracts [14].

RSD were in an acceptable range for 77 and 83 of the 93 CUPs, depending on the fortification level, with acceptable RSDs between 3.5% (sulfoxaflor, I) and 19.9% (tebufenozide, I) in the female bee samples, with 28% being below 10% (ESM Table S3). The RSD for males (10 mg) was acceptable for 87 and 89 CUPs, depending on the fortification level; RSDs within the acceptable range were between 0.09% (bentazone, H) and 17.5% (bentazone, H), with 59% being below 10% (ESM Table S3). RECs were acceptable for 72 and 89 of the 93 tested CUPs in females, with acceptable RECs ranging from 76.4% (bromoxynil, H) to 119.5% (bixafen, F). In male bee matrix, 82 and 88 CUPs fulfilled the REC criteria, RECs for acceptable CUPs ranged from 71.3% (flupyradifurone, I) to 93.8% (propamocarb, F). The linearity showed acceptable coefficients for all CUPs ($R^2 \geq$ 0.99) in females and for 89 CUPs in males (ESM Table S3). MEs were negligible for 88 (16.5% (difenoconazole, F) to 13.1% (propamocarb, F)) of 93 CUPs (ESM Table S3) in females and for 52 CUPs (−19.9% (metamitron, H) to 19.3% (bentazone, H)) in males. The LOQs ranged from 0.0002 mg/kg (propamocarb, F) to 0.08 mg/kg (pymetrozine, I) (ESM Table S3) for females and from 0.003 mg/kg (fenpropimorph, F) to 0.1 mg/kg (sulfoxaflor, I) for males. In single female bees, for 5 CUPs, the LOQs were lower than 0.001 mg/kg, 51 ranged between 0.001 mg/kg and 0.01 mg/kg, and 9 had LOQs between 0.011 mg/kg and 0.10 mg/kg. For males, 30 CUPs had LOQs between 0.001 mg/kg and 0.01 mg/kg, 14 CUPs had LOQs between 0.01 mg/kg and 0.1 mg/kg, and sulfoxaflor had LOQs above 0.1 mg/kg (ESM Table S3). Compared to our other methods, LOQs were higher in male post-pupal bees, which is probably caused by the low sample volume, which has an influence on LOQ values [33]. Lower LOQ values would be better to cover also in lower traces of CUPs present in the insects; however, our methods give first insights into the presence of CUPs in post-pupal single individuals.

On the basis of the preselection of pesticides in 2019 [28], 14 out of the 25 substances with a sales volume exceeding 100 tons in Germany [53] are also included in the validated extraction method for male bees, although we cover the fewest CUPs compared with pollen provision and flowers with this method (ESM Table S3). Our method is a quick and easy approach for detecting multiple CUP residues in individual wild bees.

Limitations—all three matrices

We started this method development with a wide range of CUPs; however, after validation, only a subset of all these CUPs were covered. The preselection of relevant compounds was conducted in 2019 [28], and method development started in 2021. Owing to the time shift and the ongoing revision of CUP approvals, some CUPs included in the methods are no longer approved for use in Germany (ESM Table S11) [54]. Continuously updating the method for approved CUPs is necessary to cover relevant or best-selling CUPs. Our methods cover a wide range of approved CUPs; moreover, CUPs that have recently been banned might also

be detected in environmental samples. It has already been shown that pesticides are detected in environmental samples beyond their use [2, 55]. Another approach would be to adapt the proposed methods for further CUPs via GC analyses. MEs have also been observed for certain compounds; therefore, further investigations of the reduction in these effects would be interesting. Although we found similar or better recoveries with the freeze-out instead of C18 or PSA, other agents could be useful to further reduce ME. MEs possibly also lead to observed incongruence in the reported LOQ values within a matrix between the compounds as well as between the matrices per compound, where also the varying sample amount has an influence.

Method application

As a proof of concept, the validated methods were applied to different environmental samples of flowers, pollen provision collected from the field, and hatching bees.

CUPs in post-pupal bees, pollen provision, and flowers

The different sample types had significantly different average numbers of CUPs per sample (ANOVA, Tukey's $p < 0.05$, ESM Table S10), with all being significantly different from the other sample types (ANOVA, Tukey's $p < 0.05$, ESM Table S10). This finding is in line with the results of a study in Michigan, USA, in which farmed blueberry flowers, honey bees, pollen collected from honey and bumble bees, and wax were sampled [56]. However, they detected more CUPs per sample than we did, possibly due to their screening for 261 CUPs and the analysis of a larger number of samples.

Across all the environmental samples, three CUPs (clomazone (H), metrafenone (F), and pendimethalin (H)) were detected in all three matrices, 25 in two of three, and 15 in a single matrix (ESM Table S7). The samples from all three matrices within the sample site and time period contained various CUP residues (ESM Table S4). Only residues of clomazone (H) and pendimethalin (H) were detected in all three matrices from the same time and site (Table 2; ESM Table S4). In a vegetable field (O3) at the beginning of June, clomazone was detected at a concentration of 0.002 mg/kg dw in flowers, 0.002 mg/kg fw in pollen provision, and 0.262 mg/kg in hatched bees (Table 1). Additionally, pendimethalin was recorded at a vegetable site in late May at 0.353 mg/kg dw in flowers, while the levels in pollen provision and post-pupal bees were below the LOQ (Table 1). Another pendimethalin detection occurred at a different vegetable site (O3) at the beginning of June, with 0.138 mg/kg dw in flowers and 0.126 mg/kg fw in pollen provision; no female bee was extracted for which reason no pendimethalin could have been detected (Table 1), as the method validation of pendimethalin failed for male bees. Pendimethalin and clomazone can be applied in vegetable cultivation (e.g. [57]). Matching detections in flowers, pollen provision, and post-pupal bees support the hypotheses of an exposure path over the crops to adjacent plants. However, contrary to expectations, most of the CUPs detected did not match the matrices, indicating that several pesticide routes can be used to contaminate the environment. The pollen provision analysed and the provision consumed by the larva may differ, even if the brood cells were built on the same day. This is particularly likely since bees also use mixtures of different pollen types with different exposure levels depending on landscape heterogeneity and flower diversity. Owing to the high variability in pollen provision, it is possible that the larval provision analysed for CUPs contained different residues than the pollen consumed by the larva. This is also in line with discrepancies between the detection of CUPs in pollen provision and flowers (ESM Table S7). Flowers were sampled right next to the field, while the bees possibly collected their pollen supply from other plants. To verify a floral uptake, a laboratory experiment would be necessary to evaluate how CUP residues applied on plants or next to flower plants are taken up to the flowers, in the pollen and exposed larvae due to pollen provision.

The concentrations (Table 2) were significantly different between the sample types (ANOVA, Tukey's $p < 0.05$, ESM Table S10). The maximal observed concentration in pollen provision was detected for the fungicide azoxystrobin (3.28 mg/kg pollen provision, ESM Table S4); contrary to

Table 1 Concentrations of clomazone (H) and pendimethalin (H) from samples taken at the same site (O1, O3) and during the same time period (late May (23.05–26.05.21) or starting in June (01.06–04.06.21)) in flowers (mg/kg dw), pollen provision (mg/kg fw), and post-pupal bees (mg/kg dw). "n.d." indicates no detection

Matrix	*n*	Type of agricultural site	Site	Time period	Clomazone (mg/kg)	Pendimethalin (mg/kg)
Flower	1	Vegetables	O3	Start June	0.002	0.138
Pollen provision	1	Vegetables	O3	Start June	0.002	0.126
Post-pupal bee	1	Vegetables	O3	Start June	0.262	No female
Flower	1	Vegetables	O1	Late May	0.006	0.353
Pollen provision	2	Vegetables	O1	Late May	n.d.	LOQ
Post-pupal bee	1	Vegetables	O1	Late May	n.d.	LOQ

Table 2 Summary table of CUP residue detection from flowers, pollen provisions collected from *Osmia bicornis*, and post-pupal single wild bees (*Osmia bicornis*) sampled in Rhineland-Palatinate in May and June 2021. Means, standard errors (SE), medians, and maximum (Max) for the numbers of CUPs detected per sample for each sample and types of agricultural sites and cumulative concentration (mg/kg) per sample for each sample type (flowers, pollen provision, post -pupal bees) and type of agriculture site (arable crops, vegetables, viticulture)

	Number of CUPs per sample				Cum. Conc. (mg/kg) per sample			
	Mean	SE	Median	Max	Mean	SE	Median	Max
Flowers								
Arable crops (*n*=7)	10.90	3.24	12	15	2.311	5.260	0.245	14.218
Vegetables (*n*=8)	11.40	2.97	11.5	15	0.394	0.487	0.162	1.199
Viticulture (*n*=5)	5.60	3.36	5	11	0.395	0.836	0.0209	1.890
Pollen provision								
Arable crops (*n*=4)	1.25	0.50	1	2	0.005	0.007	0.001	0.016
Vegetables (*n*=12)	7.33	4.36	8	15	0.393	1.170	0.047	4.101
Viticulture (*n*=19)	4.79	3.17	4	14	0.161	0.503	0.019	2.214
Post-pupal bees (O. bicornis)								
Arable crops (*n*=5)	0.40	0.55	0	1	0.005	0.01	0	0.022
Vegetables (*n*=11)	0.55	0.52	1	1	0.03	0.079	0	0.262
Viticulture (*n*=17)	0.24	0.44	0	1	0.001	0.006	0	0.023

expectations, azoxystrobin was not detected in any of the newly hatched bees (ESM Table S4). There are currently no available data on the degradation times of pesticides in pollen. Within the time of exposed larvae (May/June 2021) and post-pupal bee (March 2022), CUPs might have degraded in the bees. More importantly, CUPs can be degraded and detoxified in bees via metabolic processes [58], and microbes in the insect gut play a major role in detoxification processes [59]. Analysing the cocoon or feces might give insights if the measured CUPs in pollen provision were stored during detoxification processes in the cocoon or feces. Moreover, CUPs are transported by insects through different life stages; higher concentrations were measured in midges larvae exposed to CUPs than in adults after emergence [52]. The lower concentrations in adult midges could be explained by the excretion of contaminants during the pupation process [52], which could also explain the observed low number of contaminants detected in post-pupal bees.

Flowers

Among the 83 target CUPs, 35 (21 fungicides, 12 herbicides, and 2 insecticides) were recorded in the flower samples (ESM Table S4). CUP residues were detected in all the flower samples (100%, *n*=20). On average, 11 CUPs were recorded in flowers from arable and vegetable sites (min: 8 vegetable and 5 arable sites; max: both 15). However, average numbers were statistically greater only in vegetable sites than in flowers from viticulture sites, with an average of 6 CUPs (min: 2, max: 11; Table 2; Kruskal-Wallis test, $p < 0.05$; ESM Table S9). In a Californian study, flowers from a field border were sampled [17]; the exact number of CUPs per sample was not given, but from graphs, it can be estimated that, on average, approximately 3 CUPs were detected per flower on the basis of an analysis of 168 pesticides. The Californian study measured pollen- and nectar-producing structures isolated from flowers [17], whereas we measured the whole flower, assuming not only exposure from these structures but also exposure from direct contact, e.g. during nectar consumption. This discrepancy might explain the differences in the detected numbers.

In our samples, no statistically significant differences were measured between the cumulative concentration in flower sample from the different sites; it was 2.311 mg/kg dw in flowers from arable sites and 0.394 mg/kg dw and 0.395 mg/kg dw in flowers from vegetable and viticulture sites (Table 2, Kruskal-Wallis test, $p > 0.05$; ESM Table S9).

The presented methods for CUP extraction method developed allow the systematic study of CUPs in naturally occurring flowers, and, e.g., in flower strips, which were created to promote biodiversity. However, our results show that it is more likely that such flower strips can be problematic for insects due to the presence of CUPs.

Pollen provision of bee larvae

Overall, 35 CUPs out of our 71 CUPs included in our target method were detected: 21 fungicides, 10 herbicides, and 4 insecticides. In 94% of all collected *O. bicornis* pollen provision samples (n=33 of 35, ESM Table S4), CUP residues were detected. A significantly lower average number of CUPs was detected in pollen provision from arable sites (mean: 1.25, min: 1, max: 2, Table 2) than in pollen provision from vegetable sites (mean: 7.33, min: 0, max: 15, Table 2; Kruskal-Wallis test, $p < 0.05$, ESM Table S9). No significant differences were detected between pollen provision from viticulture sites (mean: 4.79, min: 0, max: 14, Table 2) and arable or vegetable sites (Kruskal-Wallis test, $p > 0.05$; ESM Table S9). One possible reason for this difference could be the varying management practices between arable and vegetable fields. In arable fields, crops remain on the field for longer periods of time. However, vegetable fields have greater turnover of crops during the season, which also results in more pesticide applications.

The number of CUPs in daily pollen samples from agricultural sites (average across sites: 5) aligns with daily samples from honey bee collected pollen in a fruit-growing area ([11]: average 5 CUPs). In contrast, more CUPs were detected in mixed pollen provisions collected by *Osmia bicornis* at urban sites (mean of 13 CUPs) and in orchards (mean of 17 CUPs) in the Czech Republic, where pollen provision was pooled over 14 to 23 days and 96 pesticides and their metabolites were analysed [60]. These methodological differences, along with the number of target analytes, may explain the variation in CUPs per sample. However, it is evident that CUP mixtures are present in pollen provision. Typically, CUPs are tested individually for their effects on nontarget organisms; however, research has demonstrated that *Drosophila* larvae chronically exposed to CUP mixtures (9 CUPs, no insecticide) at field-realistic concentrations exhibit widespread sublethal effects on larval development, behaviour, and reproduction [51]. It is therefore likely that the CUP mixtures detected here also have an effect on larvae even at low concentrations.

Significant differences were detected for the mean cumulative concentrations of pollen provision from vegetable sites (mean: 0.393 mg/kg dw, Table 2), which were significantly greater than those from arable sites (mean: 0.005 mg/kg dw, Table 2; Kruskal-Wallis test, p value < 0.05, ESM Table S9), and no further statistically significant differences were identified.

Post-pupal bees

Among the 65 CUPs in female solitary bees and 45 CUPs in male solitary bees, four were detected in post-pupal bees (clomazone (H, n=4), metrafenone (F, n=1), pendimethalin (H, n=6), and propamocarb (F, n=1)). On average, 0.4 CUPs were detected in post-pupal bees from arable sites, compared with 0.6 CUPs per post-pupal bees from vegetable sites and 0.2 CUPs per post-pupal bees from viticulture sites (Table 2). Overall, 14 of the 33 *Osmia bicornis* (42.4%) successfully hatched. All remaining bees (n=19) were manually removed, as it was assumed that they may have died due to pesticide exposure. CUP residues were detected in 10 of the 19 manually removed bees and in 3 of the 14 successfully hatched bees (39.4%, n=13 of 33; ESM Table S4). However, within the post-pupal bee samples, no significant differences were detected in the cumulative concentration or the number of CUPs per sample between the hatches and manually removed bees (Wilcoxon rank-sum test, p value > 0.05; ESM Table 9).

The highest concentrations (mean: 0.03 mg/kg, maximum: 0.262 mg/kg, Table 2) of CUPs were measured in bees from the vegetable sites compared with those from the arable sites (mean: 0.005 mg/kg, maximum: 0.022 mg/kg, Table 2) and viticulture sites (mean: 0.001 mg/kg, maximum: 0.023 mg/kg, Table 2); however, these observed differences were not statistically significant (Kruskal-Wallis test, $p > 0.05$, ESM Table S9). Other studies have investigated the effects of CUP residues on insects caught in the field [15, 16] and detected several CUPs, which is not surprising because these insects fly in an agricultural environment. In addition, CUPs have been detected in mixed samples of newly hatched adult aquatic insect CUPs in natural wetlands [30]. Moreover, the authors reported bioaccumulation through the food web [30]. This bioaccumulation due to the CUP load on insects is also considered a reason for CUP detection in bat guano [61].

To the best of our knowledge, this is the first study investigating CUP residues in freshly post-pupal bees, which were not exposed to CUPs in an experimental setup; for this reason, the number of CUPs cannot be compared with other data. Here, we report that newly developed insects under environmental conditions also contain CUP residues. These results indicate the widespread and lifelong exposure of insects to CUPs and the potential for bioaccumulation through the food web.

Conclusion

In this work, analytical methods for the parallel quantification of 83 CUPs in flowers, 71 CUPs in pollen provision, and up to 65 in female bees (0.02 g, in male bees (0.01 g) 43 CUPs) were developed and validated. The developed methods provided sufficient linearity and recovery with high precision. All methods are comparatively easy and inexpensive to conduct and allow the processing of many environmental samples because of fast, effective extractions. To our knowledge, this is the first target method set that allows for the analysis of multiple CUPs from different wild bee-related matrices in field-realistic small samples that can also be used for other flower-visiting insects. Our flower method (0.5 g) facilitates the study of CUPs in flowering plants using a small sample size that can be easily collected at a specific location within a limited amount of time. Although multiple pollen methods have already been established, we have developed a simple approach to analyse CUPs from the pollen of a single wild bee brood cell (0.1 g). Our unique method for extracting CUPs from individual wild bees (average dry weight female: 0.02 g, male: 0.01 g) provides a precise assessment of the exposure of wild bee individuals to CUP residues and allows to quantify the role of pesticide exposure in the observed decline in insects. Therefore, our methods enable straightforward and comprehensive analysis of the exposure of the CUP mixture to insect-relevant resources, as well as the insects themselves. The methods can be implemented in standardised monitoring and can be further developed to detect metabolites and other approved cups in future steps. The methods can be used together with the methods of soil and vegetation [28] to access CUP residues in environmental monitoring.

The application of our methods to environmental samples from an agricultural landscape revealed that flowers, pollen provisions, and new post-pupal bees contain CUP residues. The collected data provide more accurate insight into the realistic exposure of insects to CUP mixtures in agricultural areas. A meta-analysis showed that the additive effects of agrochemicals may underestimate the interactive effects of stressors on bee mortality [62]; thus, environmental risk assessment schemes fail to protect pollinators, although insects can be exposed to CUPs in various ways throughout their lives. The samples from the vegetable sites presented the most CUP residues. Therefore, insects living close to vegetable fields are potentially more exposed to CUP mixtures than to other crop types. Here, we show that insects are already exposed to CUPs before hatching and that adults start living in the agricultural landscape with a CUP burden that increases during their lifetime. Information on the presence of CUP residues in different exposure matrices for flower-visiting insects can be used to incorporate multiple pesticides into new environmental risk assessment or management approaches.

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Author contribution C.H. wrote the main manuscript text, coordinated the logistics, was responsible for project administration and data curation, and arranged the necessary permissions from relevant authorities. C.H., K.W., and M.L. collected the samples, performed the analytical analysis, and prepared the data. C.H. and K.W. analysed the data statistically and produced the figures. C.A.B. planned, acquired funding, supervised the entire project, and selected CUPs for analysis. All the authors discussed the results, commented on and improved the manuscript, and agreed on the final version.

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Data availability Data is provided within the main text and the Electronic Supplementary Material (ESM).

Declarations

Ethics approval The handling of bees in the conservation area NSG-7313-067 was performed with permission 42/553-254/475-21 by the SGD Süd, Neustadt.

Osmia bicornis pupae were purchased from www.mauerbienen-shop.de, WAB-Mauerbienenzucht, Konstanz, Germany. The experiments used larvae that had not been used in the field. For the environmental samples, *O. bicornis* individuals were used that had developed in the nest boxes with the purchased bees. The animals were handled with care.

Competing interests The authors declare no competing interests.

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Appendix III: Paper 3

Field Measurements of Pesticide Drift and Dissipation and Regulatory Exposure Assumptions

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Field measurements of pesticide drift and dissipation and regulatory exposure assumptions

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Abstract

Background The authorisation of current-use pesticides (CUPs) is based on standardised datasets that only partly reflect field conditions. Drift and dissipation are key processes determining the environmental presence of CUPs, yet field-based evidence of their fate is limited. This study aimed to provide best-case real-world drift and dissipation values after simultaneous application of one insecticide (pirimicarb) and three fungicides (bixafen, fluopyram and prothioconazole) in winter cereal fields. Application was performed under agricultural best practices, including the use of 90% drift-reducing technology and best-case weather conditions. Relative drift deposition into adjacent meadows was assessed using Petri dishes and environmental samples (bare topsoil, covered topsoil and vegetation) at distances of 1, 5 and 20 m downwind of the treated fields. Dissipation was monitored for up to 128 days. Residues were analysed via high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS). Dissipation was also evaluated with a model named MITAS (MIxture Toxicity of Application Spray series) and measured residues were compared with literature values.

Results Under a best-case application scenario, measured relative drift deposition rates exceeded benchmark values in environmental samples (topsoils and vegetation) in some cases, but not in Petri dishes. Environmental samples additionally contained residues of pesticides not applied during the experiment, demonstrating cumulative off-field contamination from other nearby sources. Dissipation in in-field soils was faster than literature-based DT_{50}. Although the investigated fields differed in soil type, no substantial differences in dissipation were observed. Dissipation in vegetation samples was in line with literature. Off-field samples showed substance-specific dissipation patterns and were often slower or not detectable.

Conclusion Overall, our results indicate that off-field transport of current-use pesticides may occur even under agricultural best practice with drift reducing technology and optimal conditions. Moreover, concentration decrease in environmental samples may be influenced by additional atmospheric inputs, which can complicate the distinction between applied and background-derived CUPs. This highlights that environmental compartments integrate multiple exposure pathways, including direct drift deposition and diffuse environmental sources. Consequently, regulatory approaches may not fully capture the complexity of real-world exposure scenarios. Field-based measurements incorporating landscape-based exposure scenarios therefore remain essential to complement regulatory modelling approaches and to ensure that environmental risk assessments adequately reflect real-world exposure scenarios.

Keywords Drift, Dissipation, Current use pesticide

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Background

Most current-use pesticides (CUPs) are synthetic chemical pesticides used to prevent, destroy, repel, or mitigate pests. However, CUPs impact not only target organisms but also non-target organisms and have been identified as key stressors for in-field biodiversity [1]. Consequently, CUPs are considered a major driver of terrestrial biodiversity decline [2]. They are primarily applied to agricultural fields, where the highest concentrations and number of residues are therefore detected. Further, CUP residues have been recorded in agricultural soils yearround [3], with compounds detected long after their initial application [4]. This leads to the presence of complex and varying pesticide mixtures that can be found in agricultural soils worldwide [5].

However, pesticide residues can also be detected yearround in adjacent meadows [3], as well as at the landscape level [6, 7]. This unintentional contamination of terrestrial non-target areas is often caused by pesticide drift during and after the application in fields [8]. Pesticide particles enter the atmosphere either during application as spray drift (primary drift) or after application through processes such as evaporation, vapour drift or wind erosion (secondary drift). Primary drift occurs during application, where all CUPs of the tank-mix are theoretically present in each spray droplet. This primary drift is influenced by the application technique and formulation rather than the active ingredients [10]. In addition, the physicochemical properties of the spray liquid [11] and added adjuvants change the drift behaviour [12]. Secondary drift, in contrast, has also been linked to the physicochemical properties of the active ingredient [13], the soil type that affects volatilisation and particle transport via wind [8]. Regarding the application equipment the nozzle type used is particularly important [14]. Nozzle design determines droplet size; with larger droplets being less prone to drift, forming the basis for drift reducing nozzles [15]. When vegetation is present, drifting molecules are likely to be intercepted by plant surfaces before reaching the soil. Meteorological conditions represent an additional key determinant of drift, particularly wind [8]. Under 'agricultural best practice' of Germany, pesticide application is recommended only under stable weather conditions, with temperatures not exceeding 25 °C, relative humidity above 30%, and wind speeds below 5 m/s [16, 17]. To minimise overspray and drift, regulatory bodies recommend the use of approved border nozzles or to alternatively switch off the outer nozzles at the field edge [18]. Pesticide authorisation procedures rely on standardised assessment approaches to predict environmentally relevant concentrations and risks of individual substances. Generic drift values used in EU regulation procedures for pesticides have been established for certain crop types and represent the 90th percentile of a large set of experimental drift measurements [19], expressed as percentage ground deposition of the application [20–22]. In these experiments, ground deposition was measured using Petri dishes placed at defined distances from treated crop fields [20].

This deposition leads to the contamination of soils, where multiple factors influence in the following the dissipation of the pesticides. Dissipation is a key process determining the environmental presence of pesticides and persistence of pesticides within a system and comprise degradative and non-degradative processes. Among the influencing factors are various abiotic factors, such as application parameters, pesticide characteristics and environmental conditions as well as biotic factors including plants and soil microorganisms [23, 24]. These factors also influence non-degradative losses, such as volatilisation, leaching and uptake by plants. On the other hand, degradation describes the biotic transformation processes of pesticides mediated by microorganisms and plants, as well as abiotic processes such as chemical and photochemical reactions [23]. Despite these processes, CUP residues often persist in the environment, leading to complex mixtures of CUPs and so-called legacy pesticides [5, 25]. Soil properties further modulate dissipation and degradation processes. Soil humidity, soil type, organic matter content and pH affect biological activity and chemical processes and thus play a central role in controlling CUP dissipation in soil [26, 27]. Dissipation rates used in regulatory risk assessment are typically determined from laboratory studies under standardised conditions [28] or semi-field studies. For regulatory purpose, only degradation within the soil is considered. Observed dissipation data are normalised to standard conditions (20 °C and pF = 2 soil moisture) and where possible, corrected for other loss processes [29]. While dissipation and degradation data for soil are widely available, comparable data for vegetation are scarce [30]. Dissipation of CUPs in plants involves processes similar to those in soils, including abiotic and biotic transformation [31], but also dilution due to plant growth.

To evaluate whether realistic drift under good agricultural practise and dissipation values included in pesticide authorisation are sufficiently protective, we sampled agricultural soil differing in their soil types and the adjacent meadows. Samples were collected after field application of four CUPs authorised for use in wheat; one insecticide (pirimicarb) and three fungicides (bixafen, fluopyram and prothioconazole). Due to the short half-life of prothioconazole (0.8 days [32]) its main metabolite, prothioconazole-desthio (PD, half-life: 25 d) [33] was also analysed. Samples were collected on the day of application to assess actual drift. To estimate the drift, Petri

dishes were placed prior to the application in the field, as well as in the adjacent meadows at 1, 5 and 20m downwind the field. Petri dishes were sampled 10 min after the application. At the same distances, topsoil samples covered by vegetation, bare topsoil and vegetation were sampled from one hour after the application. Bare topsoil was sampled, since the presence of vegetation can retain drift effects by vegetation. To evaluate the dissipation, environmental samples (topsoil and vegetation) were sampled up to 128 days after the application.

The aim of this study was to quantify pesticide drift and dissipation in a routine pesticide application under agricultural best practice conditions and to evaluate how these processes compare to regulatory worst-case assumptions. Specifically, we assessed (1) pesticide drift in relation to published benchmark values [22], (2) differences in drift between sampling matrices including Petri dishes, bare soil, and environmental samples (vegetation-covered soil and vegetation), and (3) pesticide dissipation under field conditions in comparison with standard dissipation values. In addition, (4) we investigated the influence of soil type on dissipation; and (5) evaluated the predictive performance of the MITAS model (Mixture Toxicity of Application Spray series) for time-dependent pesticide concentrations in soils.

Materials and methods

Sample sites

The study site was situated in southern Rhineland-Palatinate, Germany, within the Upper Rhine Valley, a landscape characterised by extensive agriculture and meadow systems. The northern part of the sampling area was characterized by agricultural land use, mainly arable crops and wine growing, whereas the southern part was adjacent to meadows and forested areas. The experiments were conducted in three winter wheat fields located within 2 km of each other, representing different soil types: loam (F1), clay loam (F2) and slightly loamy sand (F3) (Table 1). Soil characterisation was performed by the Agricultural Inspection and Research Institute (LUFA), Speyer, Germany. Each field bordered downwind (west to east) meadows, which were included as off-field sampling areas. The adjacent meadows were used for hay production and mowed twice a year and no pesticides or fertilisers were used according to the owners. Adjacent to F1 a flower strip was planted by the farmer with a width of 5 m, followed by grassland. The height of the vegetation at each sampling period can be found in SI Table 1.

Table 1 Soil type, pH, C/N ratio and organic matter (humus, OM) proportion of the three arable fields. The soil characterisation was performed by the Agricultural Inspection and Research Institute (LUFA), Speyer, Germany

Field	Soil type	pH	C/N ratio	OM [%]
F1	Sandy loam	6.87	10.0	3.8
F2	Clayey loam	6.28	10.3	4.2
F3	Slightly loamy sand	5.77	9.8	3.4

Pesticide application

A pesticide application was conducted in all three winter wheat fields by the same farmer. The farmer prepared a tank mixture containing one insecticide and three fungicides. The insecticide formulation Pirimior® G (Adama Deutschland GmbH, Cologne, Germany; Table 2) contains 500 g/kg pirimicarb. The fungicide Ascra Xpro (Bayer AG, Leverkusen, Germany; Table 2) includes bixafen (65 g/L), fluopyram (65 g/L) and prothioconazole (120 g/L). The products were applied in a spray solution prepared by filling the tank to a final volume of 200 L with water, corresponding to an application volume of 200 L/ha. For application, an FT 1001 sprayer (Amazonen-Werke H. Dreyer SE & Co. KG, Hasbergen, Germany) and a UF 1602 mounted field sprayer (Amazonen-Werke H. Dreyer SE & Co. KG, Hasbergen, Germany) were used. The spray boom height was automatically adjusted by an ultrasonic sensor to maintain a distance of 50 cm above the crop. Consequently, the boom followed the variable crop height during application, with winter cereals ranging from 70 cm (F2 and F3) to 90 cm (F1). Low-pressure injector eccentric flat spray border nozzles (AirMix OC 03, agrotop, Obertraubling, Germany) with a spacing of 50 cm validated for their 90% drift reducing behaviour [34] were used to minimise drift effects. The tank pressure was 2.5 bar. The application was conducted as a single spray pass with a swath width of 12 m along field sections of 95 m (F2) and 120 m (F1) adjacent to the meadow.

To ensure agricultural best practices, the application day was postponed three times because of weather conditions, e.g. wind speed or rain. Finally, on 22.05.2023, the first application (F1) started at 10:30 am, the second application on F2 started at 10:45 am, and the third started at 11:00 am. During the applications, the temperature was between 20.9 and 22 °C, wind speed was below 1 m/s from the field into the adjacent meadows (west to east). Measurements of wind speed and direction were obtained with a WinDial® wind speed indicator (Airguide, Chicago, Illinois) and subsequently validated against meteorological records from a nearby weather station.

Sampling

Samples were taken in the field (5 m), as well as in the adjacent meadow at distances of 1, 5 and 20 m from the

Table 2 Applied concentration and the measured concentration in the spray tank (200L) of the applied products (P) and physicochemical data (water solubility, vapour pressure, log P and field dissipation half-life (DT_{50})) of the applied current-use pesticide (CUP), the insecticide pirimicarb and the fungicides bixafen, fluopyram and prothioconazole, as well as for the main metabolite (M) of prothioconazole, prothioconazole-desthio and the additives (A)

Category	Product / Compound	Applied concentration	Spray tank concentration [mg/L]	Water solubility [mg/L]	Vapour pressure [Pa]	log P	Surface tension [mN/m]	Soil DT_{50} [days]	Source
P	Pirimor® G	200 g/ha		–	–	–	–	–	[35]
CUP	Pirimicarb		184	3000	4.3×10^{-1}	1.7	64.2	29–143	[36, 37]
A	Lignosulfonic acid, sodium salt			$> 5 \times 10^{5}$	–	–	–	–	[38]
A	Docu-sate sodium			8170	–	2	30.7	–	[39]
P	AscraXpro®	1.2 L/ha		–	–	–	–	–	[40]
CUP	Bixafen	65 g/L	111	0.49	4.6×10^{-8}	3.3	70.7	30.6–1235	[41]
CUP	Fluopyram	65 g/L	111	16	1.2×10^{-6}	3.3	59.4	145–347	[42]
CUP	Prothioconazole	120 g/L	919	300	$<4 \times 10^{-7}$	3.8	67	1.3–2.8	[33]
A	N,N-Di-methyl-decan-amid			340	0.11	2.46	53	–	[43]
M	Prothioconazole-desthio (PD)			50.6	0.1	3	–	7–34	[32, 33]

field. Petri dishes were sampled 10 min after the application. Sampling of soil and vegetation started 1 h after application to allow the CUPs to settle and dry. Additional topsoil covered by vegetation and vegetation samples were taken 1 day (24 h), 2 days (48 h), 4, 8, 16, 32, 64 and 128 days after application. Bare topsoil samples were taken until 8 days after application. The winter wheat was harvested between days 64 and 128 after application; therefore, in-field vegetation (crop) was sampled only until day 64. Further applications of the four selected CUPs were not conducted by the farmer throughout the entire duration of the study. Surrounding agricultural fields were not systematically surveyed for pesticide applications; however, no direct evidence, such as visual observation, indicated recent applications in nearby areas during the sampling period. Furthermore, no pesticide applications occurred on the same day as the studied application in the study areas. As the study aimed to reflect realistic field conditions, potential drift input from surrounding areas cannot be fully excluded. Samples were also collected prior to application; however, due to repeated postponements of the field experiment, sampling occurred several weeks before the actual application. Consequently, these samples were not considered representative of immediate pre-application conditions.

Nitrile gloves (Hygostar, Franz Mensch GmbH, Buchloe, Germany) and during the first 8 days after the application, protective suits (F14 (Kat 1), Adolf Würth GmbH & Co. KG, Küzelsau-Gaisbach, Germany), overshoes (Adolf Würth GmbH & Co. KG, Küzelsau-Gaisbach, Germany) and protection masks (P251-FFP2, Portwest Clothing Ltd., Thurnscoe, South Yorkshire, UK) were worn for sampling and changed between sampling points (distances), fields and days. To further avoid cross-contamination, samples were collected from the furthest sampling point and proceeding towards the field. Between the distances and fields, all sampling tools were cleaned with tap water followed by ethanol (≥ 98%, Carl Roth GmbH + Co. KG, Karlsruhe, Germany). All samples were transported from the field to the laboratory in a dark icebox filled with cool packs to avoid CUP degradation during transport (max time after sampling 4 h). After the transport, samples were frozen (- 20 °C) until further processing. All field sampling equipment was cleaned in the laboratory after each sampling day with tap water and acetone (≥ 99.9%, Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

Sampling of spray solution

An aliquot of the spray solution was taken from the tank drain valve at the bottom of the mounted field sprayer into a 50 mL PP tube (Greiner Bio-One International GmbH, Frickenhausen, Germany) to measure the actual concentration of the CUPs. The tube was wrapped in aluminium foil to prevent light-induced degradation. The spray solution was diluted 10,000-fold with water (HPLC gradient grade ≥ 99.9%, Honeywell, Charlotte, USA) before analysis of the target CUPs.

Sampling of Petri dishes

In each field (n = 3), three glass Petri dishes (Ø 120 mm, Schott AG, Mainz, Germany) were placed on bare ground and under the crop. In the meadows (n = 3), three Petri dishes were also placed on bare soil at each distance (at 1, 5 and 20m adjacent to the field). For the bare ground samples, the vegetation was removed on an area of 40 × 40 cm prior to the application. All Petri dishes were closed with a lid, wrapped with aluminium foil and put into a plastic bag in the icebox. After transport to the laboratory, each Petri dish was immediately washed in the laboratory 3 times with 2 mL of acetonitrile (MeCN, HPLC gradient grade ≥ 99.9%, Honeywell, Charlotte, USA) to dissolve the pesticides (recovery rates can be found in the supporting material Table 2). The solution was transferred as a mixed sample of the three Petri dishes per site and distances into a 20 mL glass scintillation vial (liquid scintillation vial with attached cap, WHEATON®).

Sampling of vegetation samples

The top 20 cm of vegetation was cut with stainless-steel scissors from a 20 × 20 cm section and placed in a plastic bag for collection (for vegetation height see SI Table 1). The sample consisted of a composite sample of three subsamples taken at the same distance to the field (see Fig. 1). In the laboratory, the samples were frozen with liquid nitrogen, ground in a knife mill (Grindomix GM 300, Retsch, Haan, Germany) for 1 min (3000 rpm), and dried to total dryness for 48 h in a freeze dryer (Alpha 1–4 LSCbasic, Christ, Osterode, Germany). For extraction, 1 g ± 0.01 g of the homogenised vegetation powder was weighed (ME802, Mettler Toledo, Ohio, USA; d = 0.01 g) into 50 mL tubes.

Sampling of soil samples

For the covered topsoil samples, topsoil was taken with a bulb planter (Gardena, Ø 12.5 cm) from 0 – 5 cm soil depth after removing the sward. The samples were taken at three locations per distance with a spacing of 5 m in between and the three subsamples were combined in one bag. In the laboratory, the samples were weighed (ME802, Mettler Toledo, Ohio, USA; d = 0.01 g), freeze-dried for 48 h (Alpha 1–4 LSCbasic, Christ, Osterode, Germany), sieved (2 mm mesh size, ISO 3310–1, Haver & Boecker OHG, Oelde, Germany), homogenised and weighed again. For the extraction of CUPs from topsoils, 5.00 g ± 0.01 g of soil material was weighed. For the bare topsoil samples, the vegetation was removed prior to the experiment (1 × 1 m), and topsoil samples were taken from the bare soil, similar to the covered soil samples.

Analytics for CUP residues

Validated multicomponent methods were used to extract the target CUPs and 91 additional CUPs from soil and vegetation [44]. Details on the extraction can be found in previous studies [6, 44]. In brief, 5.00 g of soil or 1.00 g of vegetation was extracted with 10 mL of acetonitrile

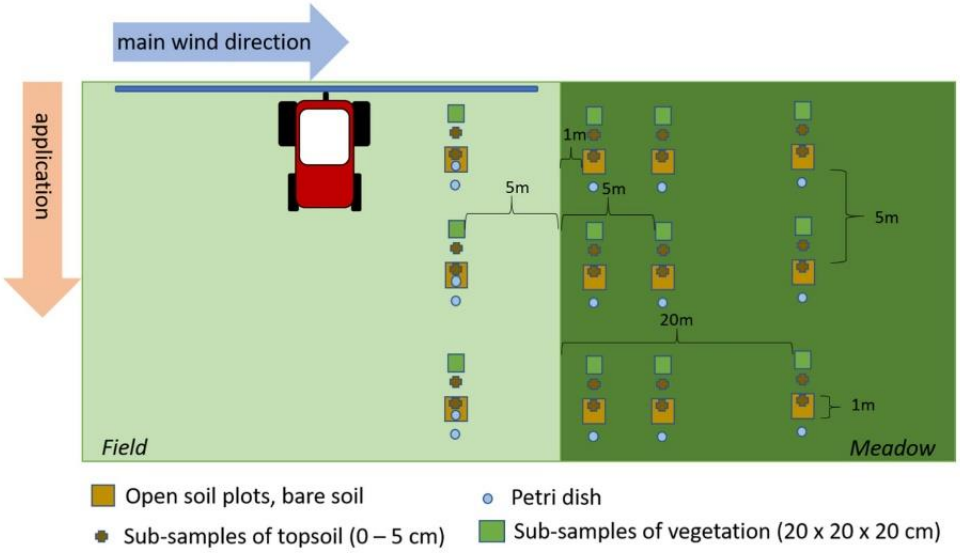


Fig. 1 Scheme of the experimental design. Subsamples with 5m in-between each sample of topsoil (brown cross) were taken from bare soil plots (brown square, 1 × 1 m), as well as from soil covered by vegetation. In-field Petri dish samples (light blue dots) were placed on bare and covered soils

(MeCN, HPLC gradient grade ≥ 99.9%, Honeywell, Charlotte, USA) containing 2.5% formic acid (CH_2O_2, HiPerSolv Chromanorm ≥ 99.0%, VWR, Radnor, USA) and either 5.00 g or 0.25 g of salt (ammonium formate, NH_4HCO_2, reagent grade ≥ 99.0%, Sigma–Aldrich, St. Louis, USA) for soil and vegetation, respectively. The samples were placed in an overhead shaker for 1 h (Reax 2, Heidolph Scientific Products GmbH, Schwabach, Germany); afterwards, the samples were centrifuged (MegaStar 1.6R, VWR, Radnor, USA, 6 min at 3000 rpm; rotor: TX-400, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and filtered (17 mm HPLC syringe filter, PTFE, pore size 0.2 µm; La-Pha-Pack, Langerwehe, Germany). The vegetation extracts were further cleaned with graphitised carbon black (GCB, Carbon SPE Bulk Sorbent, Agilent Technologies, Santa Clara, USA). For the clean-up, 1 mL of vegetation extract was added to 7.5 mg of GCB in a 2 mL Eppendorf tube. The tubes were vortexed vigorously for 60 s and the supernatant was filtered through 2 µm filters directly into the measurement vials. The MeCN solution of the Petri dishes and the spray solution were both directly injected after the above-mentioned dilution (see the Sampling section). Recoveries for the applied pesticides lay within 80–116% (SI Table 2). The extracts were analysed via high-performance liquid chromatography-tandem to triple quadrupole mass spectrometry via electrospray ionisation (HPLC)-ESI–MS/MS (HPLC: Agilent Technologies LC 1260 Infinity II series, MS/MS: Agilent Technologies 6495C, Santa Clara CA, USA). The instrumental performance and potential carry-over effects were controlled by injecting quality control and analytical blank samples, respectively. For quality control, all topsoils samples were spiked with 50 µL and all vegetation samples with 25 µL of the deuterated standard imidacloprid-D4 (98.9%, Dr. Ehrenstorfer GmbH, Augsburg, Germany) in acetonitrile (MeCN, HPLC gradient grade ≥ 99.9%, Honeywell, Charlotte, USA) (concentration: 0.05 mg/L). The solvent was allowed to evaporate for 30 min under a fume hood. A blank sample was spiked with imidacloprid-D4 after extraction (concentration: 0.05 mg/L). The peak areas of imidacloprid-D4 in the samples and of the respective blank samples were compared to check for extraction quality.

HPLC–ESI–MS/MS data were processed with Agilent Mass Hunter Quantitative Analysis software (Version 10.0., Build 10.0.707.0, Agilent Technologies, 2006–2018, Santa Clara CA, USA). CUP residues were considered positive if at least two MRM-transition products were present and if the retention time, response and quantifier/qualifier ratio (range: 70–130%) corresponded to the standard sample at a comparable concentration. The quantification of the CUP concentrations in all the samples was performed via external matrix-matched calibrations. The analyte specific limit of quantification (LOQ) for the applied CUPs was evaluated on the basis of the calibration line individually for the matrices, the values can be found in the Supporting Information (SI Table 3). Determination was based on calibration series in matrix with eight concentration levels ranging from 0.025 to 100 µg/L, considering the instrument performance on the specific measurement day. The LOQ was defined as the lowest calibration concentration were the above-mentioned criteria where fulfilled.

Statistical analyses

For the statistical analysis, Rx64 4.0.1 was used [45]. For samples with measured concentrations below the LOQ, the concentration was set to 0 for statistical analyses.

Drift

The drift was compared to the benchmark values published by the Julius-Kühn Institution [22]. According to the Ganzelmeier-Rautmann drift benchmark values used in European pesticide regulation, 90th-percentil drift after a single application on arable fields is expected to remain below 2.77% of the applied CUP rate at 1 m

Table 3 Best-estimate DT_{50} and DT_{90} [days] based on SFO-kinetics, if not indicated other, per field, substance and matrix (source: "F1- F3"), as well as the values reported by the EFSA or other studies (italics). All values are from in-field samples

Matrix	CUP	Source	DT_{50}	DT_{90}
Soil	Pirimicarb	F1, F3 covered	6.8, 13.9	22.7, 46.3
	Pirimicarb	F2 bare	4.2	13.9
	Pirimicarb	*EFSA, 2024 (Lab)*[36]	*29 – 143*	
	Bixafen	F3 covered	77.0	255.7
	Bixafen	*EFSA, 2012*[41]	*30.6 – > 1235*	
	Fluopyram	F1, F3 covered	152.5, 139.8	506.7, 464.5
	Fluopyram	*EFSA, 2013*[42]	*145 – 347*	
	PD	F1, F3 covered	60.5, 36.1	200.9, 119.7
	PD	*EFSA, 2007 (Lab)*[33]	*7 – 34*	
Vegetation	Pirimicarb	F1, F3	1.6, 0.9	5.3, 2.9
	Pirimicarb	*EFSA, 2024*[36]	*4.40*	
	Pirimicarb	*Fantke* et al. *2014* [30]	*4.60*	
	Bixafen	F1, F2, F3	0.7, 2.7, 0.6	2.4, 9.0, 1.9
	Bixafen	Wang et al. 2025	3.9 – 4.2	
	Fluopyram	F1 (HS-kinetic), F3	0.7, 0.9	11.5,3.0
	Fluopyram	*Fantke* et al. *2014* [30]	*5.5*	
	Fluopyram	*EFSA, 2013*[42]	*3.05*	
	PD	F1, F3	1.4, 0.8	4.5, 2.7

distance, below 0.57% at 5 m, and below 0.15% at 20 m [21, 22]. For the evaluation of the relative drift deposition [%], the concentrations measured in off-field samples were set in relation to the concentrations measured in in-field samples. To assess whether benchmark values were exceeded in more than 10% of cases, the proportion of exceedances was calculated separately for each distance and sampling matrix, including all applied substances and PD, as well as fields (n = 15 per distances and matrix).

Power function models To analyse CUP drift, a log–log transformation was applied to estimate a power-law decline [21] with distance downwind, with the general model form:

$y = a \cdot x^b$,

where a represents the initial value, b represents the scaling exponent describing the relationship between drift and distance, and x the distance. Concentrations were log-transformed ($\log(y+\varepsilon)$) to stabilize variance and enable multiplicative modelling of decline processes. Additional fixed effects included field and matrix type (bare and covered soil, vegetation) to account for systematic differences in drift. To test whether drift behaviour differed between matrices, interaction terms between log(distance) and matrices were included. Field differences were tested with interaction terms between log(distance) and fields. Model structures were compared using analysis of variance (ANOVA, R-package car [46]). Robust standard errors (HC3, R-package lmtest [47]) were used to assess coefficient stability under potential heteroskedasticity (Breusch–Pagan test, R-package lmtest).

Dissipation and half-life

For the environmental sample type (covered topsoil, bare topsoil or vegetation), dissipation kinetics were analysed separately from data of the three fields. The analyses included the four applied CUPs (bixafen, fluopyram, pirimicarb, and prothioconazole) and the metabolite PD.

For each field, distance, matrix, and substance, four kinetic models (single first order (SFO); hockey stick, HS; first-order multicompartment, FOMC; and double first-order in parallel, DFOP) were fitted via the mkin package [48]. Best estimate dissipation half-lives (DT_{50}) were derived from the best-fitting kinetic models using the mkin package. Models were accepted only if convergence was achieved, no ill-defined parameters were identified, the normalised root mean square error (nRMSE) remained below 30%, and residual diagnostics did not reveal systematic deviations. Model selection was further evaluated by visual inspection, and $\chi 2$ error, following the principle of preferring simpler models when adequate.

Differences between fields and sample types To assess differences between fields and sample types, nonlinear regression models were fitted for each CUP, including 'field' (three levels) and 'sample type' (covered soil, bare soil, and vegetation) as fixed additive effects. Competing models with and without these factors were compared via the Akaike information criterion (AIC). Delta AIC values indicate the support (Δ) for a given model (i) and were calculated as follows: $\Delta AIC_i = AIC_i - AIC_{min}$, with AIC_{min} as the lowest AIC value of the competing models [49, 50]. The values were evaluated based on Burnham, with a Δ value of 0–2 indicating a very strong support, 3–4 a strong support, 5–9 considerably less support and > 10 essentially no support [50].

Model for predicting time-dependent soil pesticides concentrations

"MIxture Toxicity of Application Spray series" (MITAS) is a model programmed in R [51] that assesses the exposure based potential pesticide risk in an agricultural field. For the modulation of the time dependent degradation in soil the influence of temperature on the dissipation speed of the applied pesticides is considered [52]. More detailed information can be found in a prior publication [51]. For the calculation, the applied pesticide amounts and the dissipation times (DT_{50} values) from the PPDB database [32] were used. The actual temperature data from the sampling location were applied [53], with Hamburg as FOCUS soil scenario [54]. A soil depth of 5 cm was selected.

Results and discussion

In total, 270 samples of soil, vegetation and Petri dishes were taken during this study. In 99% (n = 267 of 270) of all samples, at least one of the applied CUPs (pirimicarb, bixafen, fluopyram, or prothioconazole) or the metabolite prothioconazole-desthio (PD) was detected. In all in-field Petri dishes placed on vegetation-covered soil (n = 3) and in all Petri dishes placed on bare soil (n = 12), located either in-field or in the adjacent meadow, at least pirimicarb was detected (SI Table 4). Similarly, CUPs were recorded in all of the covered (n = 102) and bare (n = 60) topsoil samples across all distances and 97% of the vegetation samples collected (n = 90 of 93).

All applied CUPs and PD were detected in the spray solution. Based on the application volume (200 L/ha) and the application rates (200 g/ha pirimicarb (Pirimor® G), 97.5 g/ha bixafen and fluopyram and 195 g/ha prothioconazole (Ascra® Xpro)), the estimated nominal concentrations of the fungicides in the spray solution exceeded their water solubility (Table 2). This indicates that the spray solution cannot be considered a homogenous

mixture under field application conditions. Pirimor® G is formulated as a water-dispersible granule (WG) and forms a suspension upon dilution in water, it requires complete dispersion in water prior to the addition of liquid formulations [35]. Ascra® Xpro, containing the fungicides bixafen, fluopyram and prothioconazole, is formulated as an emulsion concentrate (EC) and forms an oil-in-water emulsion upon dilution. Accordingly, the spray mixture can be described as a multiphase system consisting of an emulsion (EC formulation) and a suspension of dispersed particles (WG formulation).

However, calculations of the applied rates indicated that at least of the fungicides around 100% were deposited to the bare samples (Petri dishes and topsoil, SI Table 5). However, only between 13 and 17% of the application rate of the insecticide were detected in Petri dishes and between 20 and 75% in topsoil samples (SI Table 5) indicating a high variability in topsoil samples.

Drift deposition

For relative drift deposition analyses, samples taken on the day of application were used. As all CUPs were in the same tank mix, we assumed similar resulting relative drift deposition in the Petri dishes. However, we only detected pirimicarb and prothioconazole, as well as its metabolite, in Petri dishes adjacent to the field (Fig. 2, SI Table 6). Pirimicarb was detected at all distances in Petri dishes, whereas prothioconazole and PD residues were only detected adjacent to F1 at 1m distances. These differences were unexpected, as all CUPs were initially assumed to be present within a single droplet during primary drift. Although pesticide drift has been investigated for decades, to the best of our knowledge, no studies have examined the drift behaviour of individual active ingredients within the same tank mixture. Our findings indicate that drift of CUPs within a tank mixture occurs in a compound-specific manner, suggesting that individual spray droplets do not necessarily contain all CUPs present in the tank mixture. This observed pattern is consistent with the multiphase nature of the spray mixture previously described, which may result in compound-specific differences in drift transport due to differential partitioning of active substances between emulsified and particulate phases. Regression analyses conducted separately for each CUP revealed significant distance-dependent decreases in drift-related concentrations for pirimicarb and bixafen, whereas no statistically significant trend was observed for fluopyram (SI Table 7). No significant interaction effects between matrix type or field were detected for any compound, indicating consistent spatial patterns across sampling contexts, the relatively small sample size and variability in the data may have limited the statistical power to detect subtle differences between matrices or fields.

Drift is commonly quantified as ground deposition based on residues collected in Petri dishes in drift studies [20]. For regulatory purposes, drift should be determined in area with minimum obstructions [55]. However, this does not represent the reality. For placing the Petri dishes in the adjacent meadow, we removed surrounding vegetation only locally as described in the Material and method section. The surrounding vegetation may also intercept the drift, which may limit the amount reaching the Petri dishes. This has potentially led to lower drift measurement compared to bare off-crop area assessments. In our study, drift rates measured in Petri dishes from adjacent meadows were < 1% of the field concentrations (Fig. 2, SI Table 6). The applications were performed under agricultural best practices and best circumstances, with a wind velocity below 1 m/s and with the use of authorized 90% drift-reducing nozzles [34]. In contrast, the benchmark values were evaluated as a 90th percentile of a distribution of 16 datasets [20] which could also explain the substantially lower drift rates.

Relative drift rates in environmental samples did not correspond to those recorded in Petri dishes. While Petri dishes are commonly used as a proxy for environmental drift, residues detected in environmental samples may originate not only from the experimental application but also from other sources, such as previous applications, particularly due to the systemic action of the CUPs [32, 56]. Sampling directly before the application would have help to distinguish between older residues and drifted residues. Nevertheless, this was not a possibility, as the study was conducted on a real application, and the farmer informed us only directly prior to the application. To minimise this effect, the drift rates were calculated as the ratio between the off-field and the in-field concentrations. However, the results suggest that drift rates derived from environmental samples may be of limited reliability when CUPs are applied. Bixafen and Fluopyram are widely detected substances, recorded at distances of a few km from agricultural areas [3, 6, 7, 57], suggesting off-field transport processes.

The detection of the study CUPs in environmental samples collected from meadows adjacent to the field, despite their absence in Petri dishes, may be attributed to increased effective surface area and structural complexity of vegetation in comparison to petri dishes, as well as physicochemical interactions such as sorption to plant surfaces; although this hypothesis could not be directly quantified within the scope of this study. Additionally, vegetation structure likely contributes to differences between sampling sites (Fig. 2, SI Table 6). Variations in plant composition (SI Table 1) and canopy architecture

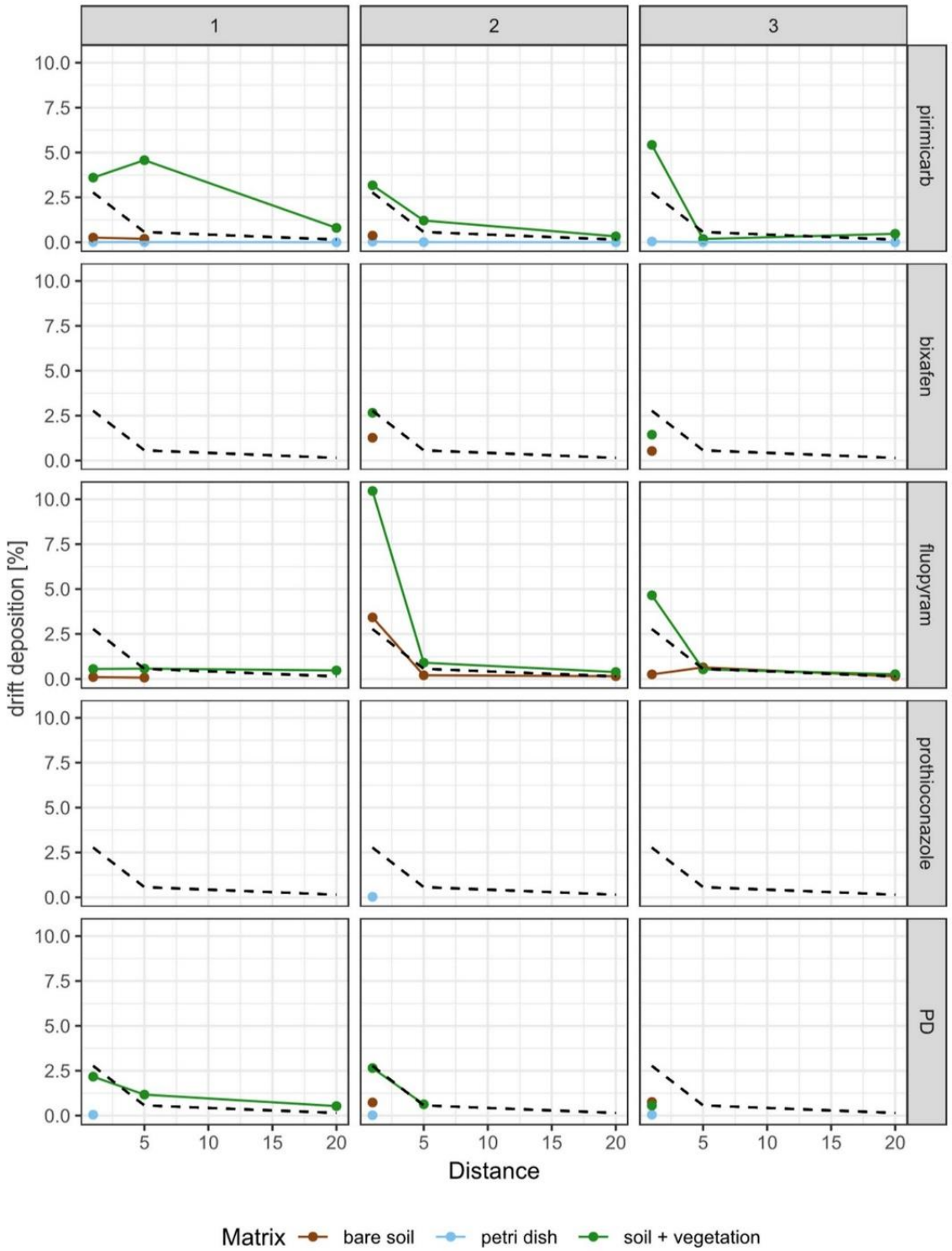


Fig. 2 Relative drift deposition [%] of the applied CUPs (pirimicarb, bixafen, fluopyram, prothioconazole) and a metabolite of prothioconazole, prothioconazole-desthio (PD), expressed as the ratio of measured off-field to measured in-field concentrations. Drift deposition or shown for Petri dishes (blue), bare soils (brown) and soils and vegetation samples (green) at the three meadows (1, 2, 3) adjacent to the treated fields. The dotted lines indicate the benchmark values for drift (dotted line, 1m: 2.77%, 5m: 0.57%, 20m: 0.15%)

can influence the interception and retention of CUPs. In particular, heterogeneous meadow vegetation may retain pesticides differently compared to more uniform field crops such as grass or cereals, potentially leading to site-specific differences in measured deposition. Moreover, vegetation therefore represents environmentally relevant exposure rather than purely aerodynamic drift. Plant structure has been shown to affect both retention and evaporation of CUPs [58]. Also from glass Petri dishes, although commonly used, it was shown that volatilisation can occur resulting in a drift underestimation [55, 59]. Nonetheless, using a different sampler, such as chromatography paper, may have led to other drift results. Nevertheless, none of the applied pesticides is particularly volatile (Table 2), making volatilisation unlikely.

Pirimicarb detections in bare topsoil were restricted to the first 5m, whereas it was detected on Petri dishes at all distances. In contrast, bixafen and fluopyram were detected in the bare topsoil but not in Petri dishes (Table 3). Prothioconazole was only detected in Petri dishes, whereas its metabolite PD was also detected in environmental samples (Table 3, Fig. 2 and 4). Drift rates in bare topsoil exceeded the threshold values for fluopyram at all tested distances in different fields (Table 3). As the benchmark values were exceeded at all distances but in different fields, this may indicate exceedances resulting from older residues with the addition of drifted concentrations.

Higher drift rates were calculated in combined residues of topsoil and vegetation samples compared to Petri dishes, with some rates exceeding the threshold values (Fig. 2, SI Table 6). For example, drift rates of fluopyram (n = 8, Fig. 2, SI Table 6) exceeding the benchmark value of 2.77% ranged from 4.65% to 6.30%. Similarly, drift rates of PD in combined topsoil and vegetation samples exceeded the benchmark values (n = 3, Fig. 2, SI Table 6), although not in Petri dishes.

These observed discrepancies in drift deposition rates between environmental samples and benchmark values indicate that regulatory scenarios may not fully represent environmental exposure after deposition under real-world conditions. For instance, herbivorous insects may be exposed to more drifted CUPs than accounted for in regulatory assessments, as they consume vegetation which contains not only the concentrations originating from primary drift. Benchmark values were exceeded in more than 10% of environmental samples (Fig. 2, SI Table 6), despite the application of the CUPs being conducted under best-case conditions with for example wind < 1 m/s and 90% drift-reducing nozzles. These findings may also help to explain frequent detections of CUPs in non-target environmental compartments during the summer months reported in other studies [3, 7]. Since routine field applications rarely occur under optimal conditions, the potential exposure of non-target sites may vary under real-world conditions. This suggests that drift estimations derived from standardised regulatory scenarios may not fully capture all processes influencing environmental exposure in non-target adjacent areas. Given these uncertainties, additional precautionary interpretation of exposure estimates may be appropriate when extrapolating from standardised drift scenarios to complex field situations. Further field studies are required to reduce uncertainty.

Dissipation

For dissipation studies, topsoil samples covered by vegetation were taken up to day 128 after application, bare topsoils for up to 8 days and vegetation samples were taken up to day 64 after application. Sampling of bare topsoil was discontinued because vegetation-free conditions could not be maintained throughout the study period and to allow assessment of CUP dissipation under realistic field conditions with covered soil rather than bare soil. Between 64 and 128 days after application, the crops were harvested, and the fields were prepared for the next crop. This included tillage and therefore incorporation of CUP residues into deeper soil layers [60]. Owing to the aim of displaying a realistic scenario, we took our last topsoil sample on day 128 regardless of cultivation. However, no in-field vegetation was available on day 128.

More complex kinetic models did not achieve better fits with one exception (Table 3, SI Table 8) than the SFO kinetics. In general, several models were not able to converge or contained ill-defined parameters. For this reason, a best estimate fit was not achievable for all individual combinations of substance, field and matrix (SI Table 8). This variability likely reflects heterogeneity in field conditions, where dissipation processes are influenced by interacting environmental factors such as soil properties, microbial activity or microclimate. Moreover, under field conditions, leaching and vertical transport cannot be excluded and therefore violate the assumption of a closed system underlying SFO kinetic, indicating that derived DT_{50} values represent apparent rather than purely degradative dissipation. In several cases, concentrations increased over time or fluctuated during the sampling period, even though the farmer did not apply further CUPs to the fields. This strikes an environmental input of CUPs throughout the atmosphere and hindered DT_{50} calculations, which are based on concentration decrease with increasing time. These findings highlight, that dissipation of applied CUPs under field conditions is strongly influenced by the surrounding agricultural practices and environmental transport of CUPs and

regulatory assumptions of dissipation of applied CUPs might not be realistic due to the widespread use of the chemicals. Especially, in bare topsoil samples, DT_{50} values could not be calculated (Table 3, SI Table 8). This may be explained by an even stronger influence of the atmospheric transported CUPs due to missing coverage. Only for the insecticide pirimicarb a DT50 value could be calculated in bare topsoil. In contrast to the fungicides, insecticides are generally less applied and might therefore be less present in the atmosphere. The DT_{50} value for pirimicarb in bare topsoil, as well as in covered topsoil, were lower than in reported DT_{50} values (Table 3). The predicted MITAS concentrations over time were higher than the measured ones (Fig. 3), suggesting a faster in-field dissipation than reported values (29 to 143 days, [36]). In the topsoils, a high variability in concentrations over time was observed between the fields, while concentrations decreased fast in bare soils from F1 and F2, the concentrations of pirimicarb in F3 remained nearly constant throughout the sampling period (Fig. 3, SI Table 4). Overall, concentrations of pirimicarb, bixafen and fluopyram, as well as PD, were detected throughout the whole study duration (SI Table 4) indicating pseudo-persistence of the pesticides in the environment.

Concentrations for bixafen in covered topsoil samples, first decreased with time, however, concentrations were higher at all sites 32 days after the application and again at the last sampling date, 128 days after the application. Highest concentration of bixafen in topsoils were measured two days after the application. The calculated DT_{50} values (77 days) was in the range of reported DT_{50} values (30.6 – 1235 days, [41]). In-field fluopyram DT_{50} values were between 140 and 153 days (Table 3) in covered topsoils. These values were at the lower end of the published DT_{50} values in the EFSA report (DT_{50} = 145–347 days [42]). MITAS model output for bixafen and fluopyram predicted lower soil concentrations (Fig. 3), indicating substance-specific underestimation of exposure. Modelled soil concentrations for prothioconazole were higher than the measured ones, which may be related to its short half-life. Importantly, there is no indication that dissipation processes were systematically overestimated by the model. Overall, the dissipation profile of prothioconazole was adequately predicted. Concentrations of its main metabolite, prothioconazole-desthio, which is known to be more persistent in soil than the parent compound [33] were not modelled.

The rapid dissipation of prothioconazole may have resulted in low recovery rates. In eight field studies referenced in the EFSA report, neither prothioconazole nor its metabolite PD were detected in topsoil samples [33]. In contrast, PD was detected in all of our in-field topsoil samples. While our samples were taken from 0 – 5 cm depths, the EFSA studies sampled the upper 0 – 10 cm. This difference in sampling depth may have contributed to dilution effects and lower measured concentrations, as vertical concentration gradients generally show decreasing pesticide concentrations with increasing soil depth [61]. DT_{50} for PD were calculatable only in covered topsoil (DT_{50} = 36—61 days, Table 3). In all other fields, PD concentrations did not decline sufficiently over the sampling period to permit DT_{50} calculation.

In general, soil organic matter and clay content are known to influence adsorption and desorption [62] and thereby reduce bioavailability. Soil type also affects the soil microbiome, which itself is a primary factor determining the persistence of pesticides in soils [63]. At the same time, CUP concentrations have been identified as a key driver of soil biota [64]. Moreover, certain CUPs may have toxic effects on the microbial communities responsible for degradation of other CUPs, thereby potentially altering dissipation dynamics [65]. Furthermore, repeated pesticide applications have been shown to alter soil microbial composition, which is associated with changes in dissipation capacity [66]. In the present study, differences in calculated dissipation between soil types were small and model comparisons did not indicate improved performance for models with soil-type specific dissipation parameters (SI Table 10). This may reflect a combination of multiple interacting process. In particular, a short-term redistribution process due to a rain event immediately after the application and subsequent dry conditions for the following two months (SI Table 12), as well as similar agricultural management practices across fields which may have reduced differences in soil conditions and associated microbial processes, although this cannot be confirmed based on the available data.

The MITAS model potentially implies a slight overestimation for rapidly dissipating single compounds and a slight underestimation for more slowly dissipating single compounds (Fig. 3). Considering the applied pesticide mixture, which is typical for agricultural soils, the predictions correspond well with the measured values (Fig. 4). The agreement between measured and predicted cumulative concentrations supports the exposure scenarios previously modelled [67] and suggests that the MITAS approach provides a suitable first-tier prediction of realistic environmental exposure under field conditions.

For off-field topsoil samples no DT50 values could be calculated. However, in covered topsoil samples from the adjacent meadows, pirimicarb was detected at all distances and up to day 128 after application (Fig. 3). Bixafen was detected 1m downwind from the field in the adjacent meadow up to day 8 after application (Fig. 3). Fluopyram was detected at all tested distances and time points in the adjacent meadows, with fluctuating

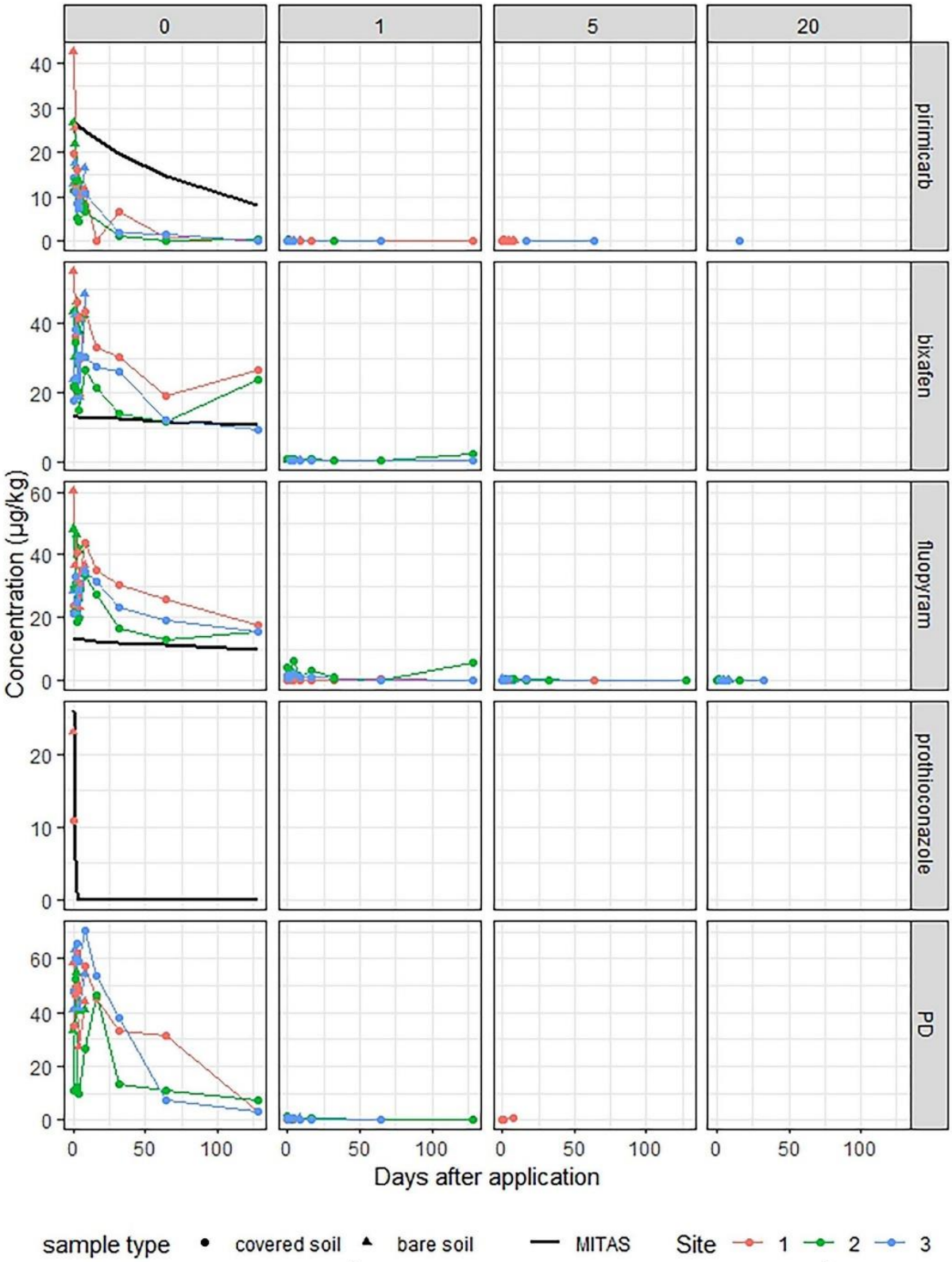


Fig. 3 Dissipation of the insecticide pirimicarb (Pirimor®G) and the fungicides bixafen, fluopryam and prothioconazole (Ascra®Xpro), as well as the metabolite of prothioconazole, prothioconazole-desthio (PD), in bare topsoils (triangle) and covered topsoils (circle). Measured dissipation patters are shown for the three sites (F1 = red, F2 = green, F 3 = blue) at the four distances (0 = in field, 1 = 1 m adjacent, 5 = 5m adjacent, 20 = 20 m adjacent to the field) and predicted in-field dissipation by MITAS is shown as black line

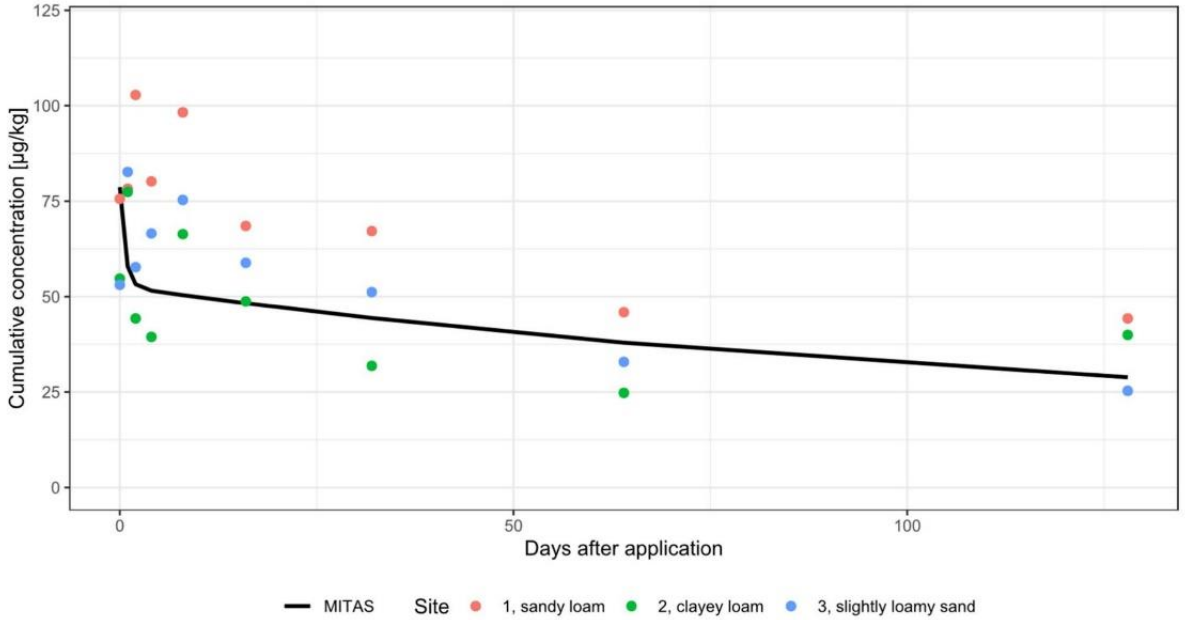


Fig. 4 Measured (dots) and predicted (black line, "MITAS") cumulative sum concentration [µg/kg] of the applied CUPs (pirimicarb, bixafen, fluopyram and prothioconazole) in covered topsoil samples over the sampling period

concentrations (Fig. 3). Prothioconazole was not detected in topsoil of the adjacent meadow, but its metabolite PD was detected at distances of 1m and5 m up to 128 days after application.

Due to the realistic situation in this study, unlike laboratory or semi-field experiments, the samples might contain initial concentrations of the applied CUPs and additional drift and deposition from the surroundings could influence the results. Pre-application sampling would have been necessary to conclusively assess this hypothesis; due to repeated postponements of the field application, soil samples collected prior to treatment were obtained several weeks in advance and therefore did not represent immediate pre-application conditions. The presence of CUPs is in line with an Europe-wide study investigated CUP residues in fields and compared the results with information about the application of CUPs; the study revealed that 79% of the detected CUPs had not been applied [25]. In a similar study, at least 32% of the detected CUPs had not been applied [68]. The authors suggest deposition from other fields or residues from previous applications. Unsurprisingly, in also in our study, up to 16 additional CUPs beside the applied CUPs and PD were detected in vegetation and 43 were detected in topsoil (for concentrations see: [69]). The detection of CUP mixtures in similar orders of magnitude in soils and vegetation has also been reported in previous studies [3, 6, 7] and probably originates beside the spray solution from previous applications, deposition, erosion or are a result of drift from adjacent fields. This is also supported by a study from Switzerland [4], where CUPs were detected in soils long after their use and it was concluded that the real degradation differs from the reported degradation. However, CUPs are also present in the atmosphere [70] and further residues detected at low concentrations in this study [69] might also originate from long-range transport. The simultaneous detection of non-applied CUPs supports the presence of background contamination. Such residues may exhibit pseudo-persistence in the environment, as degradation processes can become less efficient at low concentrations [23]. In contrast, higher initial concentrations following direct application may stimulate microbial degradation, resulting in faster initial dissipation. Similar alterations in degradation dynamics have been reported for plants when CUPs are applied as mixtures [71], highlighting that interactions between compounds can modify dissipation processes under realistic exposure scenarios.

The concentrations in in-field vegetation samples decreased over time (Fig. 5). The decline observed between the first and second sampling may be partly attributable to leaching caused by the rain event during the night following application (SI Table 12). In addition, increasing plant biomass during the growing season may have contributed to declining concentrations through growth dilution. For in-field vegetation, pirimicarb DT_{50}

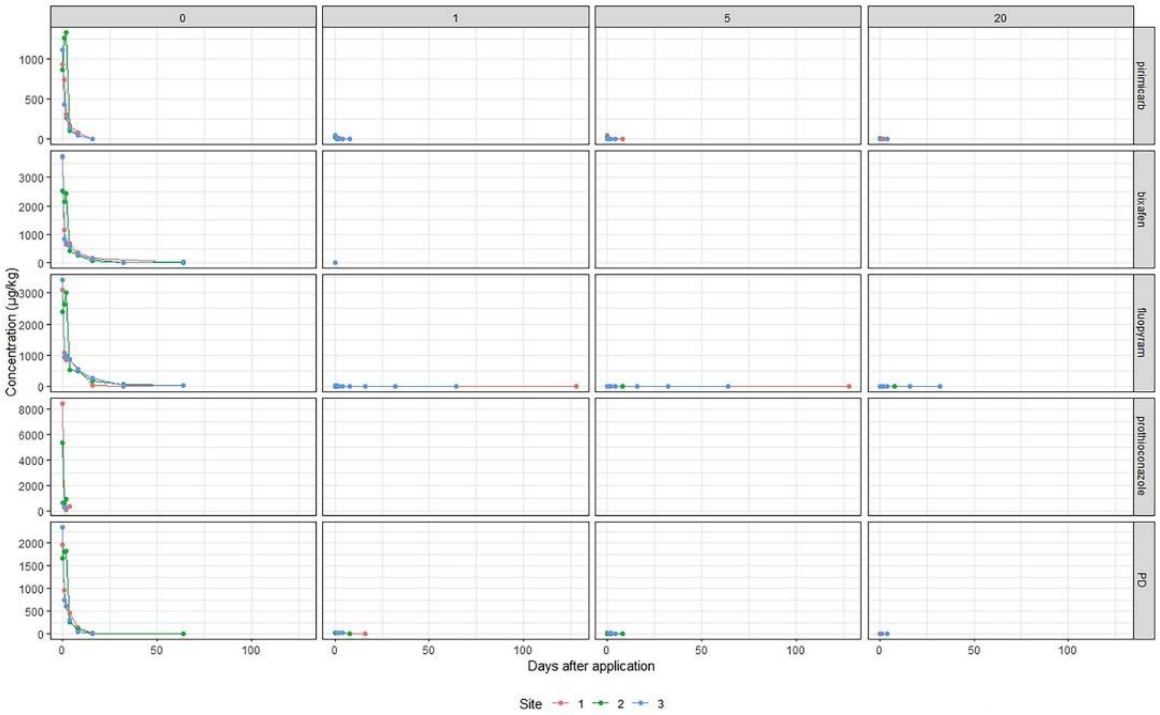


Fig. 5 Dissipation of the insecticide pirimicarb (Pirimor®G) and the fungicides bixafen, fluopryam and prothioconazole (Ascra®Xpro), as well as the metabolite of prothioconazole, prothioconazole-desthio (PD), in vegetation samples. Measured dissipation patters are shown for the three sites (site 1 = red, site 2 = green, site 3 = blue) at the four distances (0 = in field, 1 = 1m adjacent, 5 = 5m adjacent, 20 = 20m adjacent to the field)

were calculated to be 0.9 and 1.6 days (Table 4). Reported DT_{50} values for foliar applied pirimicarb were 4.4 days [72] and 4.6 days [30]. The dissipation of the fungicide bixafen was calculated between 3.89 and 4.2 days in rice [73]. In our study, DT_{50} values for bixafen in in-field vegetation were lower and ranged between 0.6 and 2.7 days (Table 3). These values are well below the assumed 90% percentile of 7.9 days [74] and are consistent with the general assumption of DT_{50} below 10 days for CUPs in vegetation [75]. For fluopyram, a DT_{50} of 5.5 days at 20 °C in plants was estimated [30]. EFSA reports a geometric mean DT_{50} of 3.1 days for plants and 4.9 days for short grasses [42]. The DT_{50} for fluopyram obtained in the present study for in-field vegetation (0.7 and 0.9 days (Table 3) lay below the reported ranges. A laboratory study reported DT_{50} for prothioconazole in wheat leaves ranging from 3.2 to 3.6 days [76]. The data was not sufficient to estimate a best fit DT50 for prothioconazole. Detectable concentrations were measured up to four (F2, F3) and eight (F1) days after the application in the cereals. The dissipation was therefore markedly shorter than the reported values. This likely reflects differences in the experimental conditions between laboratory and field settings. Similarly, in another field study, prothioconazole was not detected in foliage after application in rice, whereas DT_{50} values ranging between 2.5 and 10.1 days, were reported for its main metabolite PD, which is known to be more toxic than the parent compound [77]. However, the DT_{50} values calculated for PD in two fields of the present study were shorter, at 1.4 and 0.8 days.

Model comparisons indicated that accounting for field-specific variations in dissipation improved the explanation of the models (SI Table 10). A similar variability was reported in the previously mentioned study on pesticide dissipation in rice plants, where DT_{50} values varied by up to 8 days [78]. A literature review further supports that variability trials, rather than between substances, is typically observed [74].

In off-field vegetation, DT_{50} values could be calculated for pirimicarb and fluopyram. For pirimicarb DT_{50} ranged from 0.4 days to 0.6 days (Table 3, SI Table 9). For fluopyram, however, DT_{50} values were considerably more variable, ranging from 2.4 to 46.3 days (Table 3, SI Table 9). The pronounced variability observed in off-field samples is likely partly attributable to the comparatively low residue concentrations, for which small absolute differences can result in large relative variation. Environmental factors such as plant characteristics,

environmental conditions may have further contributed to the observed variability. It also indicates small-scale, matrix-dependent variation. As discussed for soil, also deposition from other fields might explain the slow dissipation of the applied CUPs in the adjacent meadows, leading to a continuous presence of these chemicals.

Realistic application with pesticide mixtures

In addition to the experimental CUPs applied, three other herbicides, were detected in the spray solution (SI Table 11). It can be assumed that even if the tank was cleaned after the last application or before use, which is probable since the farmer was aware that samples would be taken, residues would remain in the tank. To minimise the risk of carryover, it is necessary to clean the spray nozzles as well as the tank thoroughly. However, farmers do not necessarily carry out routine cleaning programs on a regular basis or after each application [79]. Therefore, it can be assumed that CUP mixtures in tanks can be detected regularly. Consequently, these mixtures of remaining CUPs in the spray tank are applied during the next spraying.

Conclusion

This field study provides empirical data on pesticide drift and dissipation in a real-world setting under agricultural best practice and best-case conditions. Despite the use of drift-reducing technology and favourable application conditions, calculated relative drift deposition in environmental samples adjacent to the treated fields in some cases exceeded the benchmark values [22], which are used in the authorisation process. In contrast, no exceedances were detected in Petri dishes. In this study, the contribution of the application could not be clearly separated from background concentrations. However, the results suggest that experimental drift deposition measurements may not directly reflect pesticide residues observed in real environmental compartments, which are also influenced by the occurrence of further CUPs.

Based on the collected data, DT_{50} values could not be calculated in several cases, mainly due to fluctuations in the concentrations rather than a decrease over time. Where best estimate DT_{50} values could be fitted for topsoils they were generally faster than published DT_{50} values and regulatory assumptions, likely reflecting the influence of field conditions such as temperature and environmental variability. Although the investigated fields differed in soil type, no substantial differences in dissipation were observed. In-field dissipation of the CUPs in vegetation was generally consistent with published data, falling within the range represented by the 90th percentile DT_{50} of 7.9 days [74]. Residues of most applied substances remained detectable until the end of the sampling period, indicating that rapid dissipation does not preclude persistence in environmental samples. This fact indicates pseudo-persistence, which is a continuously release of CUPs into the environment, potentially from neighbouring fields, that leads to a replenishment of dissipated residues [80]. This is particularly worrying as these CUPs inputs occur unintentionally also in non-target areas. Moreover, during the degradation processes also metabolites are formed. Those metabolites are sometimes more toxic in than the parent compound [77, 81].

Environmental samples not only contained residues of the applied active substances but also additional CUPs, confirming that areas agricultural landscapes are subject to cumulative and chronic pesticide exposure. These finding underscores that CUP drift and dissipation cannot be considered in isolation for single substances, but rather represents a continuous and complex input of chemical mixtures into terrestrial ecosystems.

Taken together, these results indicate that even under best practice and optimal conditions, off-field transport and continuous presence of CUPs occur. This indicates that standardised measurements may not fully capture the complexity of exposure patterns in adjacent non-target environments, which may be relevant for the interpretation of current regulatory drift exposure assessments. Field-based measurements that incorporate landscape-based exposure scenarios remain essential to complement modelling approaches and to ensure that environmental risk assessments adequately reflect real-world agricultural conditions.

Abbreviations

CUP	Current-use Pesticide
DT_{50}	Dissipation half-life
EC	Emulsion concentrate
Pd	Prothioconazole-desthio
WG	Water-dispersible granule

Supplementary Information

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Supplementary Material 1

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Author contributions

C.H. wrote the main manuscript text, coordinated the logistics, was responsible for project administration and data curation and arranged the permissions. C.H. and U.J. took the samples, performed the analytical analysis and prepared the data. C.H. performed the statistical analysis and produced the relevant figures. A.S. implemented and applied the model for predicting time-dependent

soil pesticide concentrations and reviewed and edited the original draft. M.R.N. contributed to the model implementation and application for dissipation prediction and reviewed and edited the original draft. C.A.B. developed the study idea, acquired funding, supervised the entire project, participated during application and sampling and reviewed and edited the original draft. C.H., U.J., C.A.B. and A.S. discussed the results. All the authors commented and improved the manuscript and agreed on the final version.

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Data availability
Data generated and analysed during the current study are included in this publishes article and its supplementary information files, as well as on Zenodo.

Declarations

Ethics approval and consent to participate
`Not applicable`

Consent for publication
`Not applicable`

Competing interests
The authors declare no competing interests.

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Appendix IV: Paper 4

Exposure of insects to current use pesticide residues in soil and vegetation along spatial and temporal distribution in agricultural sites

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Exposure of insects to current use pesticide residues in soil and vegetation along spatial and temporal distribution in agricultural sites

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Current use pesticides (CUPs) are recognised as the largest deliberate input of bioactive substances into terrestrial ecosystems and one of the main factors responsible for the current decline in insects in agricultural areas. To quantify seasonal insect exposure in the landscape at a regional scale (Rhineland-Palatine in Germany), we analysed the presence of multiple (93) active ingredients in CUPs across three different agricultural cultivation types (with each three fields: arable, vegetable, viticulture) and neighbouring meadows. We collected monthly soil and vegetation samples over a year. A total of 71 CUP residues in different mixtures was detected, with up to 28 CUPs in soil and 25 in vegetation in single samples. The concentrations and numbers of CUPs in vegetation fluctuated over the sampling period, peaking in the summer months in the vegetation but remaining almost constant in topsoil. We calculated in-field additive risks for earthworms, collembola, and soil-living wild bees using the measured soil concentrations of CUPs. Our results call for the need to assess CUP mixture risks at low concentrations, as multiple residues are chronically present in agricultural areas. Since this risk is not addressed in regulation, we emphasise the urgent need to implement global pesticide reduction targets.

Keywords Synthetic pesticide, Non-target organism, Mixture, Chronic

Pesticides are chemicals used to prevent, destroy, repel, or mitigate pests. They can also be used as plant regulators, defoliants, or desiccants[1]. The application of pesticides is recognised as the largest intentional input of biologically active substances into terrestrial ecosystems[2]. Synthetic current use pesticides (CUPs) have been detected not only in cultivated land[2–5] but also in nontarget areas[6–12] and at the landscape level[13–15].

In recent decades, a decline in aerial insect biomass was observed in both, agricultural landscapes[16] and nature reserves[17] in Germany. Many factors, such as climate change, landscape fragmentation and agricultural intensification, including the use of pesticides, are considered stressors of the observed decline[18,19]. However, in a pan-European study that measured the in-field biodiversity of plants, insects and birds, pesticides were identified as stressors with greater explanatory value than structural landscape features or fertiliser use[20].

On cultivated land, a series of CUPs are applied throughout the year. This results in the accumulation of multiple substances. This realistic mixture exposure is not consistently assessed in environmental risk assessment procedures for regulation, as a comprehensive mixture risk assessment is generally not performed. While individual tank mixtures, if listed on the label of the product with a clear name and dose rate and formulations are tested[21], the actual realistic mixtures are not considered[22]. In addition, the current insect exposure assessment does not adress soil or vegetation as sources of contamination[23], despite its acknowledged relevance[24]. Toxicological effect data on pesticide exposure in soil are available for earthworms and collembolans. Recent research has focused on evaluating the potential risks associated with pesticides for pollinators, regarding the presence of CUPs in pollen and nectar and a revised EFSA document was published[25]. Besides the fact, that bumble bees and many solitary bees build their nests in soil[26], exposure in the risk assessment by pesticides in soil is only considered through pollen and nectar contaminations via soil[25,27,28]. Moreover, there is a lack of data on the toxicological endpoints resulting from pesticides on above-ground plant material, like stems and

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leaves, despite various insects using them as nesting material, living on them, or consuming them as larvae (e.g. caterpillars) or adult (e.g. grasshoppers).

Various pesticide mixtures have been measured in recent years in agricultural soils[2–5,8,11,29,30] and non-agricultural soils[5,31] as well as in the vegetation of nontarget areas[7,9,10,32] using multicomponent analytical methods. During a study in Switzerland, agricultural soil samples with 14 years of pesticide history were investigated[33]. Their results indicate that the presence of CUPs goes beyond recent applications, forming a substantial background of various residues in the soil. However, as in most studies, the samples were collected once a year. Thus, it remains unclear how CUP residues change throughout the season, affecting the exposure of non-target organisms.

To investigate the realistic exposure of insects to CUPs in landscapes characterised by agriculture, we sampled and analysed two environmental matrices, soil and vegetation. Samples were taken from nine different agricultural sites (arable management system, vegetable management system and viticulture, each $n = 3$) separated by 0.20 to 41.8 km in Rhineland-Palatine, Germany, with an adjacent meadow to each site (for further site details see Supplementary Table S16 and S16b). We sampled every month for an entire year to be able to link exposure to the activity patterns of different insects.

The objectives of this study were (1) to quantify the seasonal variation in synthetic CUP mixtures in soil and vegetation by monthly sampling over one year; (2) to characterise spatial in-field and off-field (adjacent meadows) CUP distributions at different distances (1, 5, and 20 m from the field to the meadow); (3) to calculate the risk of realistic CUP mixture exposure for earthworms, collembolans and a surrogate ground-nesting wild bee. Our research hypotheses were that (a) CUPs fluctuate seasonally in soil and vegetation in numbers and concentration and (b) decrease with distance from the agricultural fields, with (c) far-reaching transportation in 3D (viticulture) compared to 2D (arable and vegetable) applications.

Results

CUP mixtures in soil and vegetation

Of the 93 CUPs analysed, 66 (71%, 30 fungicides (F), 23 herbicides (H) and 13 insecticides (I)) were detected in the topsoil samples (see Supplementary Table S1). Each analysed topsoil sample ($n = 468$) contained at least one CUP, 94% contained two or more CUPs and in 53% of all topsoil samples, ten or more CUPs were recorded. On average ten CUPs were recorded in soil samples. A maximum of 28 different CUPs was detected in a single topsoil sample from an arable (winter wheat) field in October (see Supplementary Table S2). The most frequently occurring substances per pesticide group in topsoil samples were the fungicide (F) fluopyram, the herbicides (H) terbuthylazine and pendimethalin) and the insecticide (I) chlorantraniliprole (Table 1). The topsoil samples revealed 349 different CUP mixture combinations with 124 mixtures containing at least one insecticide. The most common mixture of azoxystrobin (F) and fluopyram (F) in all the soil samples occurred 11 times. In the soil, the mean concentration of 29 of the 66 recorded CUPs was < 1 µg/kg. The highest mean concentration for single CUPs in topsoils was detected for metrafenone (F, 26.53 µg/kg see Supplementary Table S1). However, some CUPs, e.g. s-metolachlor (H), azoxystrobin (F) and pendimethalin (H), were detected at concentrations > 500 µg/kg (see Supplementary Table S1). The maximal concentration was recorded for pendimethalin (H, 981.18 µg/kg, see Supplementary Table S1, vegetable field (celery), in-field (−20 m), May 2021)[34]).

Sixty-two of the 93 CUPs (67%, 31 fungicides, 20 herbicides and 11 insecticides; Supplementary Table 1) were detected in the vegetation samples. Almost all vegetation samples (97%, $n = 392$) contained at least one CUP, with 89% containing two or more CUPs. The average number of CUPs recorded in the vegetation samples was seven (see Supplementary Table S2). In 30% of the samples, ten or more CUPs were recorded. Up to 25 different CUPs were measured in two vegetation samples (both celery cultivation) from vegetable fields (June and July 2021). Fluopyram (F) and chlorantraniliprole (I) are the most commonly detected fungicides and insecticides (Table 1). Prosulfocarb was the most prevalent herbicide recorded in vegetation (Table 1). In the vegetation samples, 344 CUP combinations were recorded, with 68 mixtures containing at least one insecticide. The most frequent mixture was azoxystrobin (F) and fluopyram (F), which occurred in seven samples. In the vegetation, the mean concentration of 7 CUPs of 68 was < 1 µg/kg and between 1 and 10 µg/kg for 27 CUPs. The highest mean concentration of single CUP in vegetation was recorded for fludioxonil (F, 1,349.55 µg/kg see

	Detection frequency (%)		Total mean conc. [µg/kg]		Maximum conc. [µg/kg]	
	Soil ($n = 468$)	Vegetation ($n = 442$)	Soil ($n = 468$)	Vegetation ($n = 442$)	Soil ($n = 468$)	Vegetation ($n = 442$)
Fungicides						
Fluopyram	**93.6**	**70.4**	6.04	28.79	164.62	954.42
Herbicides						
Pendimethalin	**28.2**	16.1	24.22	463.34	981.18	23,161.44
Prosulfocarb	14.1	**35.1**	0.27	4.05	0.78	28.15
Terbuthylazine	**28.2**	12.2	2.34	11.80	205.10	218.52
Insecticides						
Chlorantraniliprole	**14.3**	**10.2**	3.15	102.73	27.09	3,988.48

Table 1. Detection frequency, total mean concentration [µg/kg] and maximum concentration [µg/kg] of the three most commonly detected CUPs in soil and vegetation (in bold).

Supplementary Table S1). The maximal concentration of 67,656.10 µg/kg was measured for dimethomorph (F, viticulture, in-field (−20 m), July 2021).

Temporal CUP distribution

Temporal distribution of CUPs in soil of different crops

The number of CUP residues in topsoils remained nearly constant throughout the year. In arable fields, the CUP numbers were constant until October, when a maximum was detected, afterwards the number of CUPs slightly decreased. In-field at vegetable sites, two maxima were detected (February and October 2021, Fig. 1, see Supplementary Table S3), with a minimum in May. The same was calculated in-field at viticulture sites (Fig. 1, see Supplementary Table S3). The temporal course of the CUP numbers in topsoil samples from target areas (fields) differed only slightly among the three cultures (KL divergence ≤ 0.01, see Supplementary Table S4).

Temporal distribution of CUPs in the vegetation of different crops

Seasonal fluctuations in CUP numbers were pronounced in the vegetation (Fig. 1). In the field, the number of CUPs increased until the middle of summer. Maxima in-field from vegetation samples were detected for all managment systems in July and August (see Supplementary Table S3) and again in October for vegetables. Concerning arable and vegetable crops, similar trends were observed (KL-divergence see Supplementary Table S5) the number of CUPs decreased after the peak and remained high in viticulture (Fig. 1). In the off-field, the number of CUPs remained higher at the viticulture sites compared to the other cultivation types (Fig. 1).

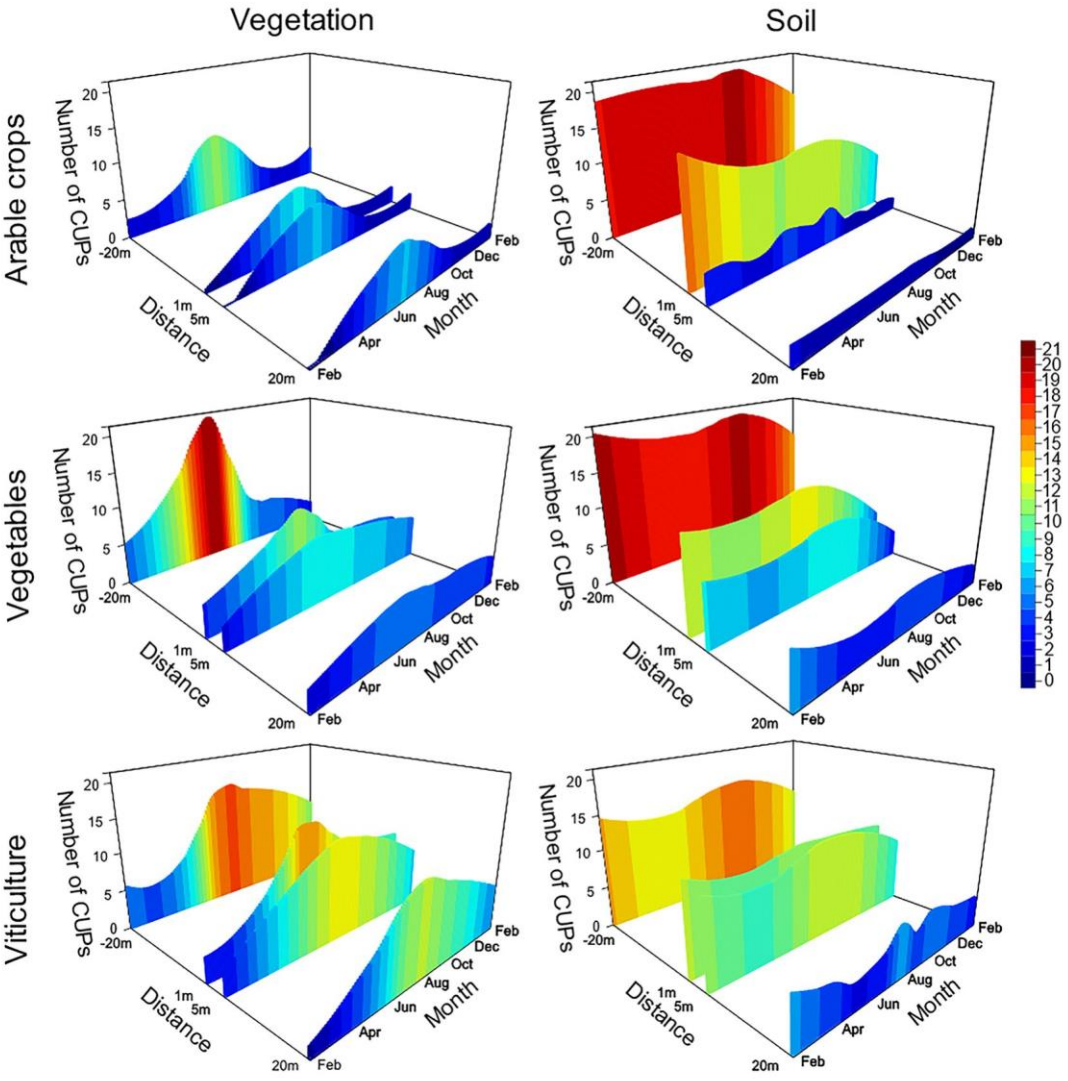


Fig. 1. Number of CUPs throughout the year in soil (left graphs) and vegetation (right graphs) from different agricultural sites (arable crops, vegetables, and viticulture) at four different measurement points (in the target area (−20 m) and off-field in an adjacent meadow (1 m, 5 m and 20 m from the field margin)) over 13 months from February 2021 to February 2022. The colours indicate the number of CUPs. The data were modelled based on the raw data with a loess smooth.

Spatial CUP distribution

An exponential function was used to fit the data and analyse the drift for the different cultivation types. A consistent decrease in CUPs was observed from in-field to meadow for all land use types in terms of soil and vegetation (Fig. 2).

CUPs in soil of different management systems

Arable crops In topsoil from arable fields, an average of 19 CUPs was detected (see Supplementary Table S6). A decrease in the number of CUPs (1 m: 13 CUPs, 5 m: 3 CUPs, 20 m: 2 CUPs, see Supplementary Table S6) was measured at the arable sites with increasing distance (b= −0.47, see Supplementary Table S7, Fig. 2). Insecticides were only recorded in the field and 1 m from the field edge, represented by 6 and 4 substances, respectively (see Supplementary S8).

Vegetables In the field, at the vegetable sites (−20 m), an average of 19 CUPs was recorded (see Supplementary Table S6). A decrease in CUPs with increasing distance was measured off-field (Fig. 2, see Supplementary Table S7: b= −0.52 and see Supplementary Table S6: 1 m: 12 CUPs, 5 m: 8 CUPs, 20 m: 5 CUPs). Over all months, eleven different insecticides were detected in the soils from the vegetable fields. At 1 m off-field, six insecticides were detected, three at 5 m and one at 20 m (see Supplementary Table S8).

Viticulture On average, 15 CUPs were detected in the in-field topsoil at the viticulture sites, decreasing to 12 CUPs at 1 m as well as 5 m and 5 CUPs at 20 m (see Supplementary Table S6). In viticulture, a greater drift into the off-field area was measured compared to arable and vegetable crops (Fig. 2, see Supplementary Table S7: b = − 0.11). The 20 m measurement point at one of the three viticulture sites was situated in a nature conservation area. During the main spraying season, a maximum of 7 CUPs was recorded at this point in August (May: 2 CUPs, June: 2 CUPs, July 4 CUPs). Overall, viticulture sites were dominated by fungicides: residues of 23 different fungicides, and two herbicides and two insecticides were detected in topsoil samples inside the vineyards (see Supplementary Table S8 and Figure S1).

CUPs in vegetation of different management systems

In 14 vegetation samples, no CUP was detected (seven samples at 20 m, six at 5 m and one at 1 m off-field). Twelve of the 14 samples without CUP detection were collected at arable sites (at 5 and 20 m), one at the vegetable site (20 m, February 2022) and one at the viticulture site (1 m, February 2021).

Arable crops On average, 7 CUPs were detected in vegetation in arable crops (see Supplementary Table S6). A decrease in CUP numbers from the cultivated area to the meadow (Fig. 2, see Supplementary Table S7: b = − 1.45) was measured. On average, 4, 4 and 3 CUPs were detected in meadow vegetation samples at 1 m, 5 m and 20 m off-field, respectively (see Supplementary Table S6). Fungicides were detected most predominantly, followed by herbicides and insecticides. Three different insecticides (acetamiprid, thiamethoxam at 5 m, spinosad and thiamethoxam at 20 m; see Supplementary Table S8) were detected in the off-field vegetation.

Vegetables On average, 11 CUPs were extracted from vegetation inside the vegetable fields (mostly crop plants as wild plants were not available in large quantities due to herbicide use; see Supplementary Table S6). At one meter in the meadow, on average, 7 CUPs were detected, 7 at 5 m and 5 at 20 m (see Supplementary Table S6). CUP numbers decreased with the distance from the field (Fig. 2, Supplementary Table 5: b = − 0.22). Fungicides were detected most predominantly. The insecticide chlorantraniliprole was detected at all sampled distances (see Supplementary Table S8). In total, eight insecticides were recorded in-field, five at 1 m, two at 5 m and two at 20 m off-field.

Viticulture On average, 12 CUPs were detected in vegetation from viticulture areas. The number of CUPs decreased slightly with increasing distance, with 10 CUPs recorded at 1 m, 10 at 5 m and 8 at 20 m (Fig. 2, see Supplementary Table S7: b = −0.15, see Supplementary Table S6). At the nature conservation area, a maximum of 16 CUPs was measured in July (May: 2 CUPs, June 7 CUPs, August 12 CUPs). No insecticide residues were detected in any vegetation sample from inside the vineyard or the adjacent meadows.

Realistic mixture exposure risk assessment

The risks of single substances were calculated for the detected CUP mixtures and are summarised in the respective risk quotients.

Collembola

For Collembola, published no-observed effect concentration (NOEC) values were available for 55 of the 66 detected CUPs. In-field, CUP mixtures posed a risk (mixture exposure risk, MRQ) in all topsoil samples for collembola, a high risk was calculated in 13% (n = 5 of 39) of the samples (mean MRQ > 1), a medium risk (0.1 < MRQ < 1) in 79% (n = 31), a low risk (0.01 < MRQ < 0.1) in 8% (n = 3) and no negligible risk (MRQ < 0.01) was calculated (n = 0) (Table 2). High-risk values (mean MRQ) in-field were calculated in July for arable fields, in October for vegetable sites and from June to August for vineyards. The risk remained at a medium level at arable and vegetable sites but at a low level in February 2021, March 2021 and February 2022 at viticulture sites. In around 20% of the off-field samples, low (22%, n = 26) and medium (20%, n = 24) risks were calculated. A high off-field risk was calculated in the meadow soil at 1 m next to the vineyard in June (see Supplementary Table S9). The off-field environment posed a risk to collembola in approximately half of the samples (44%, n = 51 of 117; see Supplementary Table S9), whereby the occurrence of risks decreased with the increasing distance. On average

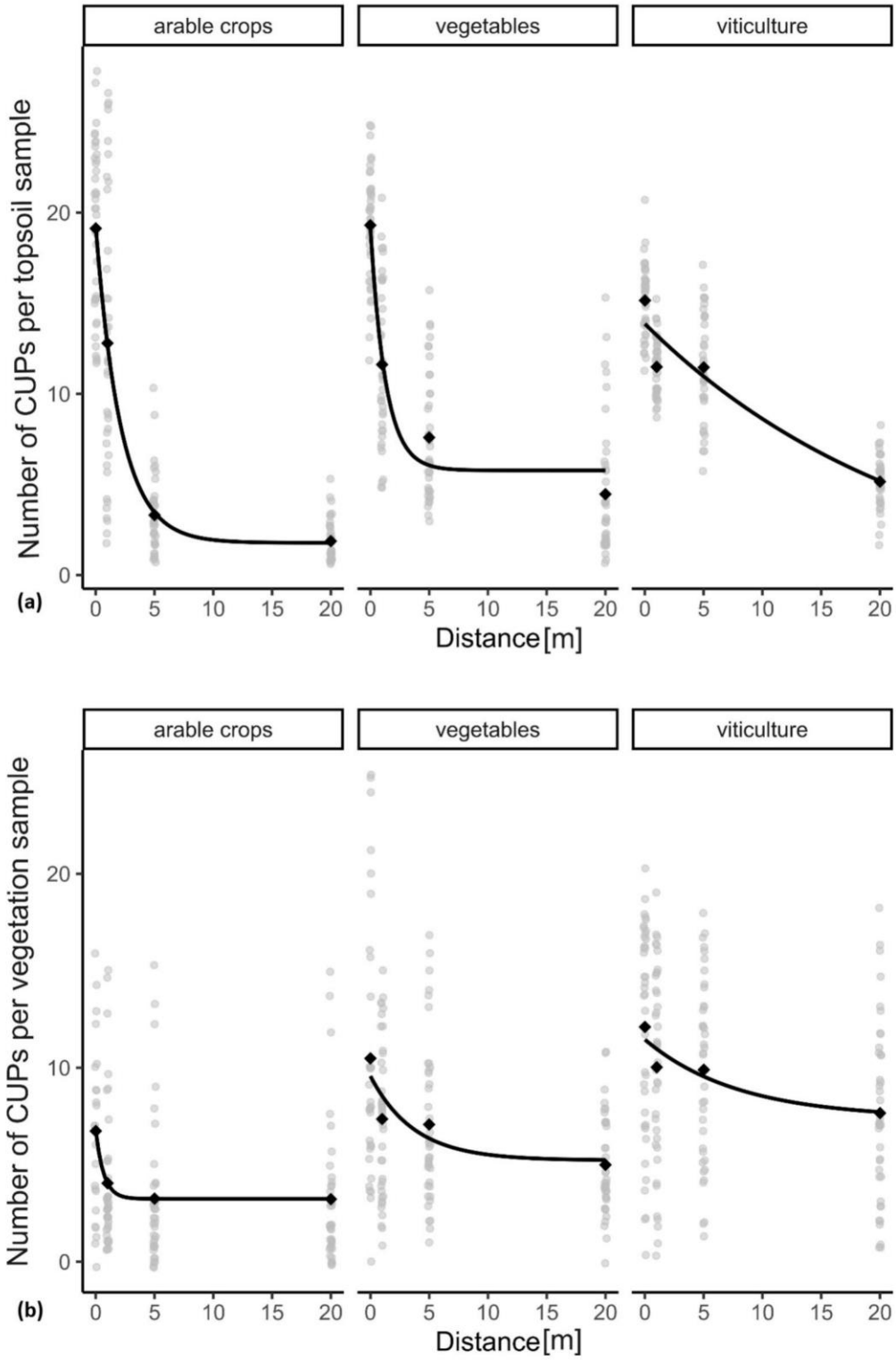


Fig. 2. Number of CUP per sample with increasing distance to the field for all investigated management systems (arable crops, vegetables and viticulture) in soil (**a**) and vegetation (**b**). CUP number per sample and distance. The curves show the exponential fit with the R-function nlsLM[35]. All samples (vegetation: $n = 442$, soil: $n = 468$) collected throughout the year are included. The in-field samples were labelled as distance 0 and the off-field samples were collected at distances of 1, 5 and 20 m in an adjacent meadow.

Month	Collembola						Earthworms					
	Arable site		Vegetable site		Viticulture site		Arable site		Vegetable site		Viticulture site	
	Mean RQT	Max RQT	Mean RQT	Max RQT	Mean RQT	Max RQT	Mean RQT	Max RQT	Mean RQT	Max RQT	Mean RQT	Max RQT
In-field (-20 m)												
Feb'21	**0.34**	**0.51**	**0.18**	**0.43**	*0.04*	*0.05*	**0.28**	**0.43**	**0.24**	**0.31**	**0.36**	**0.39**
Mar'21	**0.38**	**0.82**	**0.11**	**0.30**	*0.04*	*0.09*	**0.30**	**0.44**	**0.30**	**0.60**	***1.65***	***3.83***
Apr'21	**0.53**	**0.83**	**0.41**	***1.17***	**0.13**	**0.25**	**0.40**	**0.73**	**0.53**	**0.70**	**0.61**	***1.44***
May'21	**0.40**	**0.64**	**0.15**	**0.33**	**0.17**	**0.31**	**0.31**	**0.54**	**0.71**	***1.02***	***1.00***	***1.52***
Jun'21	**0.70**	***1.02***	**0.32**	**0.85**	***1.60***	***2.30***	**0.92**	***1.82***	***1.10***	***2.03***	***1.39***	***2.03***
Jul'21	***1.07***	***2.46***	**0.19**	**0.32**	***1.31***	***2.87***	**0.39**	**0.46**	***4.33***	***7.33***	***1.44***	***2.86***
Aug'21	**0.49**	***1.08***	**0.16**	**0.47**	***2.80***	***6.61***	**0.26**	**0.32**	**0.24**	**0.62**	***2.04***	***5.02***
Sep'21	**0.78**	***1.75***	**0.32**	**0.59**	**0.57**	**0.83**	**0.31**	**0.42**	**0.84**	***1.43***	***1.09***	***1.75***
Oct'21	**0.77**	***1.49***	***1.22***	***3.20***	**0.63**	***1.12***	**0.36**	**0.45**	***2.14***	***4.23***	***1.04***	***1.31***
Nov'21	**0.33**	**0.49**	**0.82**	***2.19***	**0.33**	**0.66**	**0.26**	**0.51**	***1.16***	***1.95***	**0.75**	***1.07***
Dec'21	**0.42**	**0.66**	**0.35**	**0.90**	**0.53**	**0.80**	**0.20**	**0.35**	**0.83**	***1.18***	***1.17***	***1.94***
Jan'22	**0.47**	**0.81**	**0.29**	**0.69**	**0.17**	**0.30**	**0.19**	**0.33**	**0.62**	**0.86**	**0.61**	**0.86**
Feb'22	**0.18**	**0.30**	**0.35**	**0.86**	*0.09*	**0.21**	**0.11**	**0.17**	**0.43**	***1.00***	**0.48**	**0.69**

Table 2. Overview of the mixture exposure risks (MRQs) calculated for collembola and earthworms based on the measured environmental concentrations (MECs) of 93 CUPs in topsoils in cultivated areas (in-field) of arable ($n = 3$), vegetable ($n = 3$) and viticulture sites ($n = 3$). The mean and maximum MRQ for both soil organisms at each agricultural site and month are shown. No risk (MRQ < 0.01, low risk (italics) 0.01 < MRQ < 0.1, medium risk (bold) 0.1 < MRQ < 1, a high (MRQ =1, bold italics) and very high risk (bold italics) MRQ > 1).

the MRQ was highest on viticulture sites (mean arable sites ($n = 156$): 0.17, mean vegetable sites ($n = 156$): 0.11, mean viticulture sites ($n = 156$): 0.26).

The maximal Risk Quotient (RQ_{max}), the risk caused by the concentration of a single CUP in the mixture, explained over all months 86% of the risk in arable in-field samples, 69% of the risk in vegetable in-field samples and 79% of the risk in vineyard in-field samples (see Supplementary Table S10). In a few instances also, single substance concentrations alone posed a high risk to collembola ($RQ \geq 1$). The presence of four CUPs, cyflufenamid ($n = 8$ of 468 topsoil samples, six in-field and two samples at 1 m), epoxiconazole ($n = 4$, in-field), cyantraniliprole ($n = 2$, in-field) and imidacloprid ($n = 2$, in-field, see Supplementary Table S11), resulted in a high risk for collembola in the topsoil samples.

Earthworms

Toxicological endpoint values (NOEC, or if not available then LC50) for earthworms were available for 64 of the 66 CUPs detected in this study. The mean MRQ ($n = 3$) for in-field samples was calculated to indicate a risk for all months and all management systems. A high risk was calculated in 31% of the in-field samples ($n = 12$ of 39), a medium risk was calculated in 69% ($n = 27$) and no low or negligible risk was calculated (Table 2, see Supplementary Table S9). No high risk (mean MRQ) for earthworms was calculated in-field at arable sites. A high risk was posed by the CUP mixtures present in the topsoils of vegetable fields in June, July, October and November and for March, May to October and December in vineyards. The percentage of off-field mean topsoil samples at risk was 50% ($n = 59$ of 117), with 1% ($n = 1$) of all off-field samples at high risk, 23% ($n = 28$) at medium risk, and 25% ($n = 30$) at low risk (see Supplementary Table S9). The number of MRQs indicating a risk decreased with increasing distance. On average the MRQ was highest on viticulture sites (mean arable sites ($n = 156$): 0.11, mean vegetable sites ($n = 156$): 0.32, mean viticulture sites ($n = 156$): 0.34). Medium risks were detected up to 20 m in the meadow of the vegetable sites.

On average ($n = 39$), 46% of the MRQ is explained by a single CUP (RQ_{max}) in in-field samples at arable sites and 62% in in-field vegetable and viticulture samples (see Supplementary Table S10). Four single CUPs posed a high risk to earthworms ($RQ \geq 1$): the fungicides azoxystrobin ($n = 2$ of 468 samples), boscalid ($n = 8$), difenoconazole ($n = 12$) and the herbicide terbuthylazine ($n = 1$) (see Supplementary Table S12).

Wild bees

LD50 values for honey bees were published for 65 of the 66 CUPs detected in this study. The mean mixture hazard quotient (MHQ) for the acute exposure scenario (2.23 g soil exposure per bee in 48 h[36]) in-field posed a hazard in 20.5% of the topsoil samples (Table 3, see Supplementary Table S13). Based on the average MHQ ($n = 3$) a hazard (MHQ > 1) was inferred from June to July on arable land and from September to February 2022 on vegetable fields. No risk was identified at vineyards. The maximum MHQ indicated a hazard for wild bees from March to July on arable soils and in February and from August to February on vegetable soils. No maximum MHQ was higher than the threshold of 1 in vineyards (Table 3). Off-field, no hazard was calculated from the measured CUP mixtures (see Supplementary Table S13).

	Arable sites		Vegetable sites		Viticulture sites	
Month	Mean MHQ	Max MHQ	Mean MHQ	Max MHQ	Mean MHQ	Max MHQ
Feb'21	0.37	0.49	0.7	**2.08**	0.05	0.05
Mar'21	0.38	**1.13**	0.01	0.01	0.09	0.18
Apr'21	0.71	**1.8**	0.35	0.52	0.04	0.07
May'21	0.41	**1.19**	0.24	0.37	0.05	0.06
Jun'21	**1.75**	**3.9**	0.08	0.12	0.08	0.12
Jul'21	**1.54**	**4.54**	0.29	0.66	0.1	0.15
Aug'21	0.33	0.94	0.64	**1.91**	0.14	0.29
Sep'21	0.25	0.71	**2.18**	**5.65**	0.09	0.1
Oct'21	0.49	0.74	**7.87**	**22.69**	0.08	0.09
Nov'21	0.29	0.52	**5**	**14.82**	0.06	0.06
Dec'21	0.63	**1.28**	**1.32**	**3.93**	0.08	0.12
Jan'22	0.31	0.68	**1.4**	**3.64**	0.05	0.05
Feb'22	0.01	0.01	**1.55**	**4.09**	0.05	0.05

Table 3. Mean mixture hazard quotient (MHQ) ($n = 3$) and the maximal MHQ were calculated based on the recorded concentrations of 93 CUPSs in in-field topsoil samples of arable, vegetable and viticulture site for solitary wild bees with a surrogate LD50 (honey bee LD50)/10[36], with LD_{50} data taken from PPDB) and an acute contact of 2.23 g of soil over 48 h. The numbers displayed in bold are MHQs > 1, indicating a lethal hazard.

Over all months, 63% of the MHQ was explained by the concentration of a single CUP (HQ_{max}) in arable in-field samples, 69% in vegetable in-field samples and 43% in viticulture in-field samples (see Supplementary Table S10). The concentrations of clothianidin (I, $n = 4$), cyantraniliprole (I, $n = 7$) and thiamethoxam (I, $n = 3$) exceeded the HQ threshold of 1 (see Supplementary Table S14).

Discussion

All topsoil samples in this study, in-field and off-field, contained at least one CUP, with an average of 10 CUPs per sample. This corresponds to other European studies, where at least one CUP was measured in 99%[2] and 98%[3] of the in-field topsoil sample. This suggests that soil in agricultural landscapes is contaminated with multiple CUPs. Methodologically, the number of detected CUPs depends on the number of CUPs that are analysed if targeted methods are used. For instance, in a French study, 111 different CUPs were included in a target analysis of topsoil samples, of which 67 different substances were detected[8]. We analysed 93 different CUPs and detected in total of 71 CUPs, 66 in topsoils and 62 in vegetation. In addition, Glyphosate and AMPA, were among the most frequently detected CUPs in the French study, with 83% and 70% detection rates, respectively[8]. These results correspond to a European-wide study[11], which also detected a mixture containing AMPA and glyphosate (included in 25% of pesticide combinations[11]), to be the most common CUPs in soil samples. Therefore, we can assume that glyphosate and AMPA are very likely also occurring in our soils. In contrast, the most abundant CUP in our soil samples was fluopyram (in 94% of all topsoil samples), which was also detected in 69% of topsoil samples in a French study[8] but was not included in the target substances in other studies[3,11]. Fluopyram is marketed as a fungicide in Europe but is used as a nematicide in tropical crops such as bananas[37], therefore it has negative effects on nematodes and available data indicate a negative effect of a fluopyram metabolite on plants[38] and soil arthropods[39]. Since our analysis covers only a part of the authorised CUPs in Germany, it is fair to assume that even more CUPs are present in in-field soils, resulting in even greater mixture complexity. We detected almost 350 different CUP mixture combinations in topsoil and vegetation by analysing 93 target compounds, with the most common mixture being detected only on eleven occasions in soils and seven in vegetation samples. The amount of combinations makes it impossible to assess the mixture toxicity risk organisms face in reality if this would ever be a regulatory aim. At least one insecticide was detected in 124 mixtures in soils and 68 mixtures in vegetation. In our target method, fewer insecticides than fungicides and herbicides are included, which may explain the lower frequency of insecticides observed in the mixtures. We assume that more mixtures contain insecticides than were observed in this study, as some insecticide groups, such as pyrethroids, can only be detected with different analytical methods. This limitation in detection capacity is an important caveat of our study, as it does not cover all authorised pesticides. To establish pesticide monitoring in the terrestrial environment, all registered molecules, as well as their metabolites or transition products, should be included in a target method and incorporated in the current proposal for an EU Directive on Soil Monitoring[13,40].

A comparison of the detected substances with the application data provided by the farmers would have constituted an interesting research approach. However, as our study was conducted with actual farmers rather than model farms, we were dependent on their willingness to participate, which prevented the collection of such data.

Comparing cumulative concentrations across studies requires caution due to the use of different analytical methods, as underlined in a previous study[5] and discussed above. Individual concentrations can be cautiously compared, considering variations in quantification limits, extraction methods and sampling times. It should also be considered that high variability in CUP concentrations has already been observed in soil samples[41].

Nonetheless, the highest concentration measured in our agricultural topsoil samples was detected for pendimethalin (981.18 µg/kg, vegetable, cultivated area, May 2021). In French arable soils, pendimethalin was detected at a concentration of 923 µg/kg in spring[5] and at a concentration of 1,115 µg/kg in January[8]. In another study, a maximum concentration of pendimethalin (310 ng/g) was recorded after the field season[4]. The comparison shows that our data are quite comparable with those of other European studies when individual concentrations are considered.

The sampling time differed between studies; some were conducted before and others after the field season[42]. It was unclear whether the exposure data recorded in the studies were representative of the entire year or whether they were just displaying a specific moment. Our data show that a single sampling event is not representative of the whole year. Due to the differences in February 2021 and February 2022, there may likely be differences in exposure between years. In light of this, comprehensive, long-term monitoring is essential to gain a full understanding of the situation and its temporal dynamics. In the topsoils, we recorded a high baseline of CUPs throughout the year with various maxima. This result is not consistent with our hypothesis of seasonal variation, which was based on the timing of pesticide application. It might be influenced by multiple factors: CUP residues from deeper soil layers, which have been preserved there, can enter the topsoil through tillage. Crop senescence and harvest residues can also distribute CUP residues into the soil[43]. Moreover, the soil is also treated with CUPs before and after cultivation, mainly with herbicides to prepare the field for cultivation. During the agricultural season, the soil is covered by vegetation and most of the applied substances remain, depending on the development stage, on the crop foliage and only a small fraction reaches the in-field soil[44]. We therefore observed higher concentrations in vegetation than in soil. For example, the highest measured concentration in soils (pendimethalin, herbicide, 981.18 µg/kg) was orders of magnitude lower than the corresponding vegetation sample (23,161.44 µg/kg, vegetable site, in-field, May 2021), although the latter was not the highest concentration measured in vegetation. The highest concentration of an individual CUP in vegetation was recorded for dimethomorph (fungicide, 67,656.10 µg/kg, viticulture sites, in-field, July 2021), as the corresponding concentration in the topsoil was 6.86 µg/kg[34]. The maximal mean concentration for a single CUP was measured at 26.53 µg/kg metrafenone in topsoils and 1,349.55 µg/kg fludioxonil in vegetation, again showing the higher concentrations in vegetation.

To our knowledge, this is the first study investigating the annual variation of terrestrial CUP concentrations. To analyse our data an approach of loess smoothing and a Kullback-Leibler (KL)-divergence was chosen to capture the courses of the year as accurately as possible. By the use of usual complex models like for example GLM/GLMM limitations have quickly been reached with our data set, e.g. due to the unknown distribution of the data or the missing linear relation between the predictor and the explanatory variable. The chosen approach with loess smoothing and KL-divergence was also used in a recent ecology study where time series were tested[45]. In our analysis, we used the data from all three repetitions from one management system to model the general temporal trends, applying both a loess fit and an exponential function to the combined data. The loess fit smoothing over the repetitions, revealing the underlying temporal patterns[45,46], while the exponential model is used in current pesticide emission models to estimate the drift[47,48]. Neither of these methods explicitly accounts for plot-specific variability, as they do not include random factors. CUP contamination in vegetation showed the hypothesised seasonal fluctuations, with an average of 7 CUPs per sample and a maximum of 25 CUPs in two single samples (in-field (−20 m), vegetable: celery, June and July). The observed fluctuations in the loess fit indicate a substantial deviation from the surrounding months. Although the Loess method does not provide p-values or formal statistical significance, the robustness of the fit implies that this peak reflects a meaningful difference, rather than random variation[49]. Grass samples from public areas in the Vinschgau Valley taken four times per year showed statistically significant changes in CUP contamination between seasons[9]. The highest CUP numbers were detected in early summer (May/June), while we measured maxima in late summer (July and August). The Vinschgau Valley is dominated by apple orchards that are treated earlier than the crops that we studied. However, fungicides were the dominant group in both studies.

Vegetation, both in-field and off-field, is more likely than soil to be contaminated by wet deposition and spray drift[50], as soil is covered for a long period by vegetation. Additionally, vegetation takes up systemic CUPs from the soil[51], which may account for some of the CUPs that were measured at lower concentrations. However, dilution effects caused by plant growth, as well as off-field mowing and grassing, can strongly influence the concentrations measured in vegetation samples. These factors present inherent challenges in field experiments, as they are difficult to quantify and cannot be easily accounted for in statistical analyses.

CUP residues were detected in the majority of the samples throughout the year. We show that the soils of agricultural areas, as well as those of the immediate surroundings, are loaded with mixtures of different CUPs throughout the year, indicating potential chronic CUP exposure for insects. Nevertheless, we also detected maxima and minima for the number of CUPs (Supplementary Tables 5, 8). Most maxima were measured in autumn, which can be explained by the accumulation of CUPs throughout the field season.

It is still unclear what effects the presence of up to 28 different CUPs (measured in in-field soil in arable field in October) has on insects and other organisms[52]. Although meadows were less contaminated than cultivated, in-field areas, mixtures of multiple CUPs were detected all year round.

We assumed that the number of detected CUPs decreased with increasing distance to the field. The decrease was tested by exponential functions, commonly used to model pesticide drift (e.g[53]). The hypothesised decrease in CUP numbers was detected for topsoil samples for all three management systems but less so for vegetation samples. In the latter, similar numbers of CUPs were recorded at different distances in the meadow. The number of CUPs was highest in the meadows adjacent to the vineyards and was less different from the in-field values than for the other management systems. This might be explained by the different ways of application; the 3D application, which is used for room cultures like viticulture or in orchards, to cover the canopy, leads to higher

drift effects[54]. This finding is consistent with our hypothesis that there are differences between the various application types.

While the study design includes three repetitions per management system, with a total of 18 plots (9 in-field and 9 off-field), we acknowledge that this level of replication may still be considered limited, particularly in the context of the high variability between sites and management systems. The relatively low number of repetitions could make it challenging to fully account for all sources of variation. However, this design was a balance between practical constraints and the need to capture variability across different conditions. Conducting larger-scale studies with more repetitions would have been ideal, but such an approach is often constrained by logistical factors. Future studies could benefit from increasing the number of repetitions or expanding the range of covariates considered in the analysis to better capture the complexity of the system. On the other hand, we collected a large, unique dataset of soil and vegetation samples, comprising approximately 1,000 samples over the course of a year. In this field of research studies so far comprised e.g. 75 soil samples in Hvězdová et al. 2018[2], 317 soils in Silva et al. 2019[11], 180 soils in Pelosi et al. 2021[5], 71 vegetation samples in Linhart et al. 2019[7] or 96 vegetation samples in Linhart et al. 2021[9]. Our comprehensive dataset has the potential to benefit other researchers in a number of ways: It can be used as a benchmark for comparative studies, as a resource for secondary analyses, or as a foundation for model development and long-term monitoring efforts.

In all soil samples ($n=468$), CUPs were detected, while in 14 of 442 vegetation samples, CUPs were not detected. Most uncontaminated samples were recorded at the greatest distance from the field in 2 D crops (20 m off-field: $n=7$ of 14 and 5 m $n=6$). In a study investigating CUP residues in vegetation at similar distances, no CUPs were detected in 5 of 7 vegetation samples at their furthest distance (>10 m), in 2 of 8 at 5–10 m, and all field edge samples (9 samples) were contaminated[32]. In a landscape characterised by agriculture, CUPs are recorded not only at the edge of the agricultural field but also at further distances, as shown here and also in previous studies[7–10,13,32,55].

Insects that live in an agricultural landscape are therefore potentially exposed to CUP mixtures not only by visiting or living in the fields themselves but also in the surrounding area, e.g. adjacent meadows. We assume that the main source of CUP contamination in vegetation samples from the meadow was drift during applications[10,56] or via volatilisation and/or dust from soil and plants after application, due to the physical-chemical properties of the pesticides, which are known to facilitate such processes (e.g., volatility, adsorption to particles). The pesticide concentrations in vegetation from public playgrounds were already positively correlated with the amount of rainfall and wind speed during application times[7]. However, air deposition[57] and rain events[58] are also inputs of CUPs, with transport from sources several kilometers away.

In our study, we also addressed the risk of CUPs to insects. However, the publicly available toxicology data basis is not sufficient, as there are no toxic endpoints for soil insects. Furthermore, as there is no risk assessment for herbivorous insects (e.g. grasshoppers or caterpillars) required, there are no toxic endpoints for oral insect exposure. Therefore, no risk from the consumption of contaminated vegetation could be calculated. Nevertheless, for approximately half of the CUPs detected in topsoils, the concentrations were below 1 µg/kg, while the concentrations for individual CUPs in vegetation samples were higher (7 of 61 CUPs with concentrations below 1 µg/kg). Concentrations in vegetation samples reached up to 67,656.10 µg/kg (dimethomorph, F, viticulture sites, in-field, July 2021). While insecticides were not detected at all off-field distances in topsoil, they were detected at all distances in vegetation samples from arable and adjacent meadows, indicating that the risk to insects is likely to be even higher here. These findings highlight the need to develop and analyse risk approaches for oral uptake via vegetation.

We showed that, on average, soils from the field posed a high to medium risk throughout the year for earthworms and collembola, except in three months for collembola (mean MRQ, Viticulture, February 2021, March 2021, February 2022, low risk). Furthermore, we showed that samples from meadows also posed a risk to collembola and earthworms, although the risk decreased with increasing distance to the agricultural field for all land use types. However, as nontarget areas also harbour chronic risks for at least earthworms[5,8], potential exposure-free and low-risk habitats for organisms are becoming increasingly scarce. As toxicological endpoints for springtails were not available for all measured CUPs, the risk might be even higher. The bioavailability of residues and the potential for synergistic effects of CUP mixtures need to be further investigated. Recently, a meta-analysis showed that the risk of pesticide exposure, assuming additive effects, underestimates the interaction effect on bee mortality, which in most cases is synergistic[59]. In our study the in-field risk was to some extent explained by single CUP concentrations, explaining on average 43 to 86% of the mixture risks, depending on the management system. Also, some single CUPs represented a high risk. The reduction or even avoidance of CUPs that trigger a high risk might improve the risk situation for non-target soil organisms. We identified eleven fungicides (azoxystrobin, boscalid, cyflufenamid, difenoconazole, dimoxystrobin, epoxiconazole, fluopicolide, fluopyram, metrafenone, myclobutanil, penconazole) five herbicides (clomazone, ethofumesate, pendimethalin, propyzamide, terbuthylazine) and five insecticides (chlorantraniliprole, clothianidin, cyantraniliprole, imidacloprid, indoxacarb) that contribute substantially to the measured risks. However, by changing CUP applications and substances the risk of other substances might increase.

There is currently no approach that addresses pesticide residues in soils as an exposure route for insects. Most wild bees nest in the soil[25,60]. Ground-nesting bees are in direct contact with the soil and larvae are exposed not only to the residues in the soil but also to the pollen. An in-field assessment of the risk to ground-nesting bees from systemic insecticides was recently performed[36]. Due to a lack of information, the authors used toxicological endpoints for honeybees to determine the risk for adult females to experience soil contact during nest building[61]. The ground-nesting honey squash bee (*Peponapis pruinosa*) used in this approach does not exist in Europe, but several other ground-nesting bees are known to build similar nests in agricultural soils in Europe[62]. *Peponapis pruinosa*, it is similar in size to common ground-nesting bees such as *Andrena flavipes*, which have been observed building nests in European agricultural fields[62].

Our wild bee risk evaluation shows that in-field, the CUP residues lead to an MHQ > 1 in arable and vegetable fields but not in viticulture. This may be because insecticides are not or less commonly used in the latter. At the vegetable sites, an MHQ > 1 was calculated from August to February, when most of the ground-nesting adult bees were no longer active. However, larvae and pupae overwinter in soils and are therefore assumed to be continuously exposed to potential pesticide residues, although their dormant phase within cocoons may on the other hand reduce their direct exposure generally uptake of pesticides can currently only be assumed as no data are available. We consider this aspect of the risk assessment as a supplementary evaluation of the potential exposure of soil-living insects and their development stages. The calculated risk may not lead to direct mortality in organisms but sublethal effects that occur at lower concentrations result in population-level effects e.g. due to lower reproduction[63], especially with chronic exposure over longer time spans.

We detected different insecticides in topsoils and vegetation at all sites and in vegetation at arable and vegetable sites. Chlorantraniliprole (I) was the most commonly recorded insecticide in both matrices. It is a newer insecticide used for tree fruits, grapes, vegetables, and maize[64]. Chlorantraniliprole reduced the locomotor activity of honey bees after 72 h of exposure[65] and was already detected in honey bee pollen[66], supporting our results. Pendimethalin, which together with terbuthylazine was the most frequently reported herbicide in topsoil samples, can affect the immune response and reduce the lifespan of honeybees[67]. The herbicide with the most detections in vegetation, prosulfocarb, was also detected with the highest frequency in German urban and agrarian bumblebee samples[68]. Prosulfocarb (H) and terbuthylazine (H), were also detected in all insect samples from 21 nature conservation areas in Germany, as were the fungicides fluopyram and azoxystrobin[52]. These results are consistent with our findings that insects are particularly frequently exposed to these substances.

Current risk assessments are based on the effects of single substances on individual organisms. Furthermore, it is assumed that there is sufficient time after application for populations to recover to previous levels[22]. However, our results show that there are no CUP-free periods. It is therefore improbable that recovery can be achieved in a realistic situation. In particular, in the field soil, high numbers of CUPs are recorded all year round, resulting in chronic exposure of soil insects or life stages of insects, e.g., larvae or pupae. High-resolution sampling over a year enables us to obtain CUP data about the activity of insects. In the vegetation, there is a maximum amount of CUP detected in the summer months, when many insect larvae feed on the plant material (e.g. Lepidoptera larvae) or use the vegetation for oviposition. When exposed to CUP residues as larvae on host plants, effects on longevity and wing length were observed in emerging butterflies[69]. Insecticide-treated plants were found to experience reduced pollination and limited to a single egg-laying event by moths, resulting in a 40% decrease in their offspring[70]. For adult wild bees (*Osmia cornuta*) exposed to sublethal concentrations of a mixture containing only one fungicide and one insecticide, a reduction in reproduction rate was observed, ultimately leading to a decline in population size[71,72]. Furthermore, insecticides have been shown to have carryover effects on wild bees[73] and other nontarget insects[74].

Conclusion

This investigation provides to the best of our knowledge the first temporal resolution of CUP residues in topsoil and vegetation in different management systems and adjacent non-target areas as common structural features in agricultural landscapes. In particular, complex mixtures of multiple CUPs were present throughout the year. They were not only recorded in the cropping area (in-field) but also in neighbouring, non-target meadows at distances of up to 20 m. This constant presence of multiple CUPs in agricultural landscapes leads to a chronic mixture of exposure to insects and other non-target terrestrial organisms.

The documented CUP exposure pattern is particularly worrying considering that most of the adult insects are mainly active in summer when the greatest number of CUPs are detected in vegetation. It has already been shown that flying insects collected in nature conservation areas are exposed to multiple CUPs[52]. In the topsoil, on the other hand, a high number of CUPs are present throughout the year, so adult insects or larvae living in the soil are chronically exposed to several CUPs at low concentrations. It is important to note that the constant presence of CUP mixtures is not part of the regulatory environmental risk assessment procedures for pesticide regulation. The remit of authorities at the EU and national levels lies in the risk characterisation of single substances and only occasionally are mixtures addressed in formulated products with up to four CUPs. We therefore urge authorities to ensure that chronic contamination with complex CUP mixtures occurring in reality is incorporated into authorization procedures and risk assessments, to prevent an unprotected environment. While risk management and mitigation strategies exist, pesticides are nevertheless detected even in areas far removed from agricultural landscapes[55].

Furthermore, together with the demonstrated landscape-scale distribution of CUP mixtures during the application time[13], our analysis demonstrates that this large-scale contamination is to be expected throughout the year. Additionally, the complexity of mixtures and the calculated risks are supposed to be even higher, as our analysis included only a fraction of the CUPs used in Europe (93 of the most commonly used out of 281). In the context of the observed insect declines, it is crucial to recognise, identify and reduce the risks posed by CUPs to insects. As mixture exposure is not addressed in the EU regulation, only reductions in pesticide risk, as included in national targets in the EU (e.g. Germany's National Action Plan aiming to minimize the risk associated with pesticide use[75], or France's commitment to reducing the use and risks of pesticides[76]) or globally by the UN until 2030[77], can change the currently observed declines. Together with conversion targets to reach organic practices in 25% of the agricultural area[78], where no synthetic pesticides are used, the required transformation of agriculture is started and exposure of organisms reduced, allowing the recovery of ecosystem services for a truly sustainable agriculture.

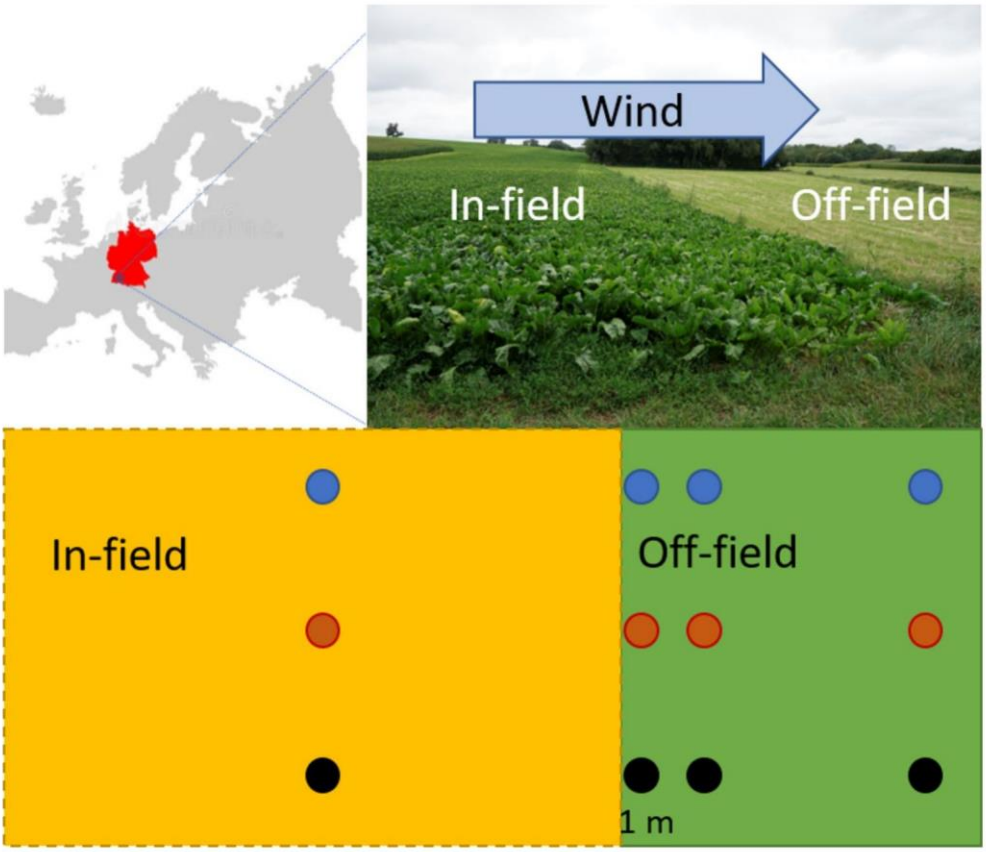


Fig. 3. Study area and layout of the field and meadow: The study was conducted in the southern Rhineland-Palatinate (blue star) in Germany between Bad Bergzabern (district Südliche Weinstraße) and Schwegenheim (district Germersheim). On each of the nine sites, a field and an adjacent meadow were studied. Soil and vegetation samples were taken 20 m from the field border in the cultivated area (field, yellow square) and at three distances (1, 5 and 20 m from the field border into the meadow) in the meadow (green square). For each sample three subsamples (blue, red and black dots) were taken with a 5-meter distance between them, leading to four samples per month and sample site. Samples were taken monthly from February 2021 to February 2022.

Materials and methods

Study sites

This study was conducted on cultivated areas and meadows downwind of the main wind direction (SW) in southern Rhineland-Palatinate in Germany (Fig. 3). Sampling occurred monthly between February 2021 and February 2022 (see Supplementary Table S15), to cover a whole year, including the most important application periods, but also the seasons with less intensive pesticide applications. The study included nine fields with each having an adjacent meadow. Conducting fieldwork across a wider range of sites was not feasible because sampling required visits every four weeks, involving considerable travel time between locations. Ensuring consistent, high-quality sampling within the available resources was prioritized over increasing the number of fields. Furthermore, the process of recruiting participating farmers was challenging, as only a limited number were willing to contribute to the study. Requesting detailed pesticide application records on top of this could have further discouraged participation. Consequently, the study focused on assessing broader residue patterns rather than site-specific application regimes, balancing practical limitations with research objectives. Our farms were also not part of a model farm network and therefore their applications reflect a common and realistic conventional pesticide management.

We chose three fields for each management system (arable farming, vegetable cultivation, vineyards). We investigated three fields for the arable managing system representing crops typical of the Rhenish crop rotation system[79]: one field with a cereal crop (winter wheat) and two with leafy crops (maize, sugar beet). This selection reflects the sequences in the cropping system. While the replication at the field level was limited, the fields were chosen to represent different cropping practices commonly observed in the region. The vegetable fields followed a succession planting pattern (e.g., fennel followed by celery, then radish, Supplementary Table 16). Although multiple fields of the same crop per management system, such as wheat, would have been preferable to reduce variability, the selection was constrained by the fields that were available for inclusion in the study. Given the limited number of fields accessible, which included a mix of crops for arable farming and vegetable cultivation, the study aimed to reflect the diversity of agricultural practices encountered in real-world conditions and to capture variations in agricultural practices and pesticide application methods. Arable farming typically involves

long-term crops that remain in the field for extended periods, with pesticides applied in a two-dimensional (2D) spraying. In contrast, vegetable cultivation is characterised by quicker crop rotations, also involving 2D pesticide applications. Vineyards, on the other hand, involve a permanent crop, with pesticide applications occurring in three dimensions (3D).

We included an adjacent meadow to each field as a non-target area for comparison. All the farms followed conventional management practices involving the use of synthetic pesticides. Pesticides were applied to the fields according to good agricultural practices based on the specific crop. Six of the nine meadows were used for long-term hay production, two others were privately owned or municipal long-term green spaces and one was a nature conservation area. All meadows were only mowed once or twice during the year and consisted mainly of grasses. The sampling was conducted with the explicit permission of the landowners. All landowners were informed, that the information they provided is used for publication. In order to ensure that landowners remain anonymous, no further details of the exact locations are given.

Sampling and sample preparation

Sampling was carried out at each of the nine sites, at four sampling points each (20 m in the cultivated field (in-field, target area), 1 m, 5 m and 20 m from the field edge into the meadow (off-field, non-target area), Fig. 3). Samples were taken at all sites ($n = 9$) and distances ($n = 4$) each month ($n = 13$). At all distances and months, it was possible to take soil samples, resulting in 468 topsoil samples, while not at all in-field samples vegetation was growing at all months, resulting in a total of 442 vegetation samples. For each sample, a composite sample was obtained from three subsamples collected at a distance of 5 m from each other (Fig. 3) to get representative samples.

For the soil samples, after removing the vegetation and root layer, 0–5 cm topsoil was taken with a flower onion planter tool (Gardena, Ø 12.5 cm) at three locations per distance and stored in a sample bag combining the three subsamples. The topsoil samples were weighed in the field, transported to the laboratory and stored in a freezer (−20 °C). The samples were then freeze-dried for 48 h (Alpha 1–4 LSCbasic, Christ, Osterode, Germany), sieved (2 mm, standard-compliant test sieve, ISO 3310-1, Haver & Boecker OHG, Oelde, Germany) and homogenised. For the extraction of CUPs from topsoils, 5.00 g ± 0.01 g of soil material was weighed (ME802, Mettler Toledo, Ohio, USA; d = 0.01 g).

For the vegetation samples, cultivated plants and "weeds" (if available) were collected in the field. For the off-field samples, differing grasses and herbaceous plants were collected in the meadow. The samples were hand collected at three locations (see above) using laboratory gloves, filling half of a 1 L freezer bag with the three subsamples. The above-ground vegetation (approximately upper 20 centimetres) was taken as vegetation samples. The combined vegetation samples were first air-dried overnight in the laboratory at room temperature, frozen (−20 °C) and subsequently freeze-dried for 24–48 h, depending on the moisture of the sample. The vegetation samples were ground in a knife mill (Grindomix GM 300, Retsch, Haan, Germany) for 2 × 1 min at 3000 rpm to avoid heating. The ground vegetation was stored in small freezer bags at −20 °C until extraction. For extraction, 1 g ± 0.01 g of vegetation powder was weighed into 50 ml tubes. The reason for using less matrix for vegetation samples compared to soil samples is primarily due to the availability and volume of material collected during the sampling process. Nonetheless, the precision is the same with also a low error margin for vegetation samples. The relative error of about 1% for vegetation would not significantly influence the CUP concentrations and can be neglected.

All tools used in the field were cleaned with ethanol (≥ 98%, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and tap water e.g. the onion planter tool and in the lab with acetone (≥ 99.9%, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and tap water. All other materials e.g. sieves were also cleaned with acetone and tap water.

Analytics for CUP residues

Validated multicomponent analytical methods[80] were used to extract 93 CUPs (36 fungicides, 36 herbicides, 21 insecticides) from soil and vegetation. Details on the CUP selection can be found in[80], the most frequently-used herbicides and fungicides as well as all insecticides from 2016 to 2017 recorded by the Julius-Kühn Institute were selected[81]. By 2021, three fungicides, five herbicides and eight insecticides had been withdrawn from the market, in total 77 of the 93 substances were approved in 2021. In brief, 5.00 g of soil or 2.00 g of vegetation was extracted with 10 ml of acetonitrile (MeCN, HPLC gradient grade ≥ 99.9%, Honeywell, Charlotte, USA) containing 2.5% formic acid (CH_2O_2, HiPerSolv Chromanorm ≥ 99.0%, VWR, Radnor, USA) and either 5.00 g–2.00 g of salt (ammonium formate, NH_4HCO_2, reagent grade ≥ 99.0%, Sigma–Aldrich, St. Louis, USA) for soil and vegetation, respectively. The samples were placed in an overhead shaker for 1 h (Stuart drive Rotator drive STR4, Cole-Parmer, Vernon Hills, USA); afterwards, the samples were centrifuged (MegaStar 1.6R, VWR, Radnor, USA, 6 min at 3000 rpm) and filtered (17 mm HPLC syringe filter, PTFE, pore size 0.2 µm; La-Pha-Pack, Langerwehe, Germany). The vegetation extracts were further cleaned with graphitised carbon black (GCB, Carbon SPE Bulk Sorbent, Agilent Technologies, Santa Clara, USA). For the clean-up, 1 ml of vegetation extract was added to 7.5 mg of GCB in a 2 ml Eppendorf tube. The tubes were vortexed vigorously for 60 s and the supernatant was filtered through 2 µm filters directly into the measurement vials.

The extracts were analysed by high-performance liquid chromatography-tandem to triple quadrupole mass spectrometry via electrospray ionisation (HPLC)-ESI-MS/MS (HPLC: Agilent Technologies LC 1260 Infinity II series, MS/MS: Agilent Technologies 6495 C, Santa Clara CA, USA). The instrumental performance and potential carry-over effects were controlled by injecting quality control and analytical blank samples, respectively. For quality control, all the samples were spiked with 50 µL of the deuterated standard imidacloprid-D4 (98.9%, Dr. Ehrenstorfer GmbH, Augsburg, Germany) in acetonitrile (MeCN, HPLC gradient grade ≥ 99.9%, Honeywell, Charlotte, USA) (concentration: 10 mg/L). The solvent was allowed to evaporate for 30 min under a fume hood. A blank sample was spiked with imidacloprid-D4 after extraction (concentration: 10 mg/L). The peak areas of

imidacloprid-D4 in the samples and of the respective blank samples were compared to check for extraction quality.

HPLC-ESI-MS/MS data were processed with Agilent Mass Hunter Quantitative Analysis software (Version 10.0., Build 10.0.707.0, Agilent Technologies, 2006–2018, Santa Clara CA, USA). CUP residues were considered positive if the MRM-transition products were present and if the retention time, response and quantifier/qualifier ratio corresponded to the standard sample at a comparable concentration (range: 70–130%). The quantification of CUP concentrations in all samples was performed using external matrix-matched calibrations. Detailed information on the limit of quantification (LOQ) and limit of detection (LOD) is available at[80]. For the total number of detected CUPs in the samples, residues < LOQ were also included. However, for the calculation of total CUP concentrations, only residues > LOQ were used.

Risk assessment

We used the measured topsoil CUP concentrations to calculate a conservative cumulative chronic risk for earthworms and collembola, as well as a hazard quotient for wild bees. For these calculations, only soil contact exposure was considered, as no other exposure routes were included. To date, there are no recommendations for approaches that include potential synergistic effects[5], so additive approaches were chosen. For HQ calculations, we used an assessment factor of 10 for comparability with other recent studies[5,8,12,82]. The Hazard Quotient (HQ) approach was used to assess the risk of ground-nesting solitary bees[36]. There is limited availability of toxicological endpoints for insects and approved risk assessment approaches. We chose the proposed approach to determine the risk posed to ground-nesting insects from soil based on the concentrations detected. The honey bee lethal effect endpoints (median lethal dose, LD_{50}) were used because honey bees are the current regulatory standard and are considered to be an appropriate proxy for all bee species[83]. We used the contact acute LD_{50} values reported in the "Pesticides Properties DataBase" (PPDB[84]). A surrogate solitary bee contact LD_{50} (honey bee LD_{50}/10) was also used. The matrix of a ground-nesting bee (*Peponapis pruinosa*) was exposed as described previously[36]. This wild bee does not occur in Europa. However, other wild bees such as *Andrena flavipes*, *Halictus scabiosae* or *Systropha planidens* also nest in soil, are similar in size and occur in or near agricultural areas in Europe[85,86]. We estimate a similar exposure to *Andrena flavipes* whose nests are similar to *Peponapos pruinosa* which is common in Europe and was detected in European agriculture fields[62].

It was estimated that female *Peponapis pruinosa* bees have contact with 2.23 g of soil during their burrowing activity for nest construction during one day[36]. Only soil contact exposure was addressed in the calculation.

The HQ was then calculated for each CUP residue in each topsoil sample as follows:

$$HQ = \frac{(Matrix\,exposure\ [g])\left(MEC\ \left[\frac{ng}{g}\right]\right)}{\frac{honey\,bee\,LD_{50}}{10}\left[\mu\frac{g}{bee}\right]}) = \frac{(2.23\,g)\left(MEC\ \left[\frac{ng}{g}\right]\right)}{\frac{honey\,beeLD_{50}}{10}\left[\mu\frac{g}{bee}\right]}) \quad (1)$$

Individual HQ values from one topsoil sample were further summed to obtain the mixture hazard quotient (MHQ, Eq. 2) in the topsoil sample.

$$MHQ = \sum HQ \quad (2)$$

An HQ > 1 indicates a potential lethal hazard occurring from the CUP residues. The concentration of residues was given in µg of active ingredient (a.i.) per kg of soil matrix, which equals ng (a.i.)/g (matrix); the amount of exposure to the matrix was reported in g of matrix per bee and the LD50 values were reported in ng a.i. per bee.

Additionally, the risk posed by cumulative concentrations of CUPs for earthworms was calculated following the methodology proposed by[12] and applied in recent studies[5,8,12,82,87]. The chronic no-observed-effect concentration (NOEC) for earthworms (*Eisenia fetida*) and collembola (*Folsomia candida*) from the PPDB were utilised. If a no-observed effect concentration (NOEC) was not available, reports to the European Commission and reports of the European Food and Safety Authority were used. If no NOEC was available, then the lethal concentration where 50% are killed (LC50) was used. The toxicity assessment for earthworms was based on 55 NOECs and 9 LC50 values, while for collembola, all were based on the NOEC ($n = 55$; see Supplementary Material Table S18). The NOEC was divided by an assessment factor of 10[5,8], or the LC50 by an assessment factor of 1000[5], to compute the predicted no-effect concentration (PNEC)[88]. The risk quotient (RQ) was then individually calculated for each CUP as the difference between the PNEC and the measured environmental concentration (MEC) in each soil sample (Eq. 3, SI, Eq. (1) to (7)).

$$RQ = MEC/PNEC = MEC/(NOEC/AF) \quad (3)$$

Subsequently, the mixture exposure risk (MRQ, Eq. 4) was calculated for each topsoil sample.

$$MRQ = \sum RQ \quad (4)$$

The MRQs were further categorised into four risk groups: negligible risk, MRQ < 0.01; low risk, 0.01 < MRQ < 0.1; medium risk, 0.1 < MRQ < 1; high risk, MRQ = 1, very high risk ≥ 1[89]. The same procedure was used for the data from the springtail *Folsomia candida*.

Statistical analysis

For the statistical analysis, Rx64 4.0.1 was used[90]. For samples with values below the LOQ, the concentration was set to 0 for statistical analyses. To avoid multicollinearity issues, principal component analyses (PCAs) (package: stats, function: princomp[90]) were constructed. To identify local CUP minima and maxima during the year, a linear model was built to subtract the trend for the number of CUPs of the year from the number of shorter-term fluctuations; furthermore, a peak detection algorithm was used[91,92]. This enables us to compare CUP residues and insect development stages. The algorithm used a threshold delta value to determine when a peak (maximum or minimum) was reached. The algorithm iterates through a given vector, updating the maximum or minimum candidate based on the current value and the threshold. If a current value deviated by more than a delta from a previous extremum, a new extremum was detected. When a peak was identified, its position and value were recorded. To increase the overall detection rate, the delta was set to 0.3. A delta of 0.3 was considered appropriate as it allows for the detection of a broader range of relevant signals without significantly compromising the reliability of the results. We acknowledge that it is somewhat subjective. In general, delta values are often set based on the dataset, to increase the overall peak detection rate (e.g. in[93]). The peak finder is only used to standardize the interpretation of the peaks and does not influence the measured and fitted peaks themselves. We constructed a generalised linear model (GLM) to see, whether the predictors have a significant influence on the number of CUPs (see SI GLM section). For the analyses of the courses over the year, the following approach was used, which has recently been applied to quantify differences in time series patterns across ecological systems[45]: A loess smooth was used to obtain a coherent progression over the year and distance to the field margin[49]. The loess smoothing was further cross-validated, for further details on the loess smoothing, see the SI Loess smoothing section. Individual loess smooths were compared with the Kullback-Leibler (KL) divergence. KL-divergence is used to measure differences in distributions or patterns[45,94]. It can be considered as a measurement of information[95].

The KL divergence was calculated as follows:

$$KL\left(P\|Q\right) = \sum P\left(P\right) * \log 2\left(P\left(P\right)/P\left(Q\right)\right) = H\left(P,Q\right) - H\left(P\right) \tag{5}$$

where $H(P,Q)$ denotes the joint entropy of the probability distributions P and Q and $H\left(P\right)$ denotes the entropy of probability distribution P[96]. KL divergence, while using direct yearly progressions, allows a comparison of curve patterns (for the SI, see the KL divergence section).

The distance to the field margin was fitted with an exponential function with nlsLM from the minpack.lm package[35]. It was assumed that the measurements in cultivated areas (−20 m) also represented the concentration at the field margin (0 m). This assumption was made because the −20 m measurement is considered representative of the general in-field concentration. Measurements at the field border (0 m) would likely not be fully representative, as the field edges are often subject to lower pesticide rates due to drift reduction techniques, which aim to minimize off-target drift. Rather than interpreting the 0 m measurement as the CUPs at the field edge, it is more accurate to consider it as indicative of the in-field measurement.

The exponential function was set up as:

$$y = a * e^{(b*x)} + c \tag{6}$$

where a is the amplitude, b is the base, c is the constant term, y is the response variable and x is the explanatory variable. In this study, the value a served as a vertical scaling factor, describing the amount of additional CUP detected at the margin in comparison to higher distances. The value b indicates the decay speed (if negative). Lower b values represent faster decay. The mean of the respective subset of the − 20 m distance was used for c, describing the background concentration that the function should have reached.

Data availability

Data is provided within the manuscript, the supplementary information and under https://doi.org/10.5281/zenodo.11517560

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Competing interests

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Appendix V: Paper 5

Current-use pesticides in vegetation, topsoil and water reveal contaminated landscapes of the Upper Rhine Valley, Germany

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Current-use pesticides in vegetation, topsoil and water reveal contaminated landscapes of the Upper Rhine Valley, Germany

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Non-target areas in agricultural landscapes serve as invaluable refuges for organisms and safeguard biodiversity. This research aimed to examine the landscape-scale distribution of Current Use Pesticides (CUPs) in the Upper Rhine Valley in Germany, a region characterised by intensive agriculture in the valley and bordered by forested lower mountain regions. We sampled vegetation, topsoil, and surface water at 78 non-target, off-field sites during the pesticide application season in 2022. The sites were located in six 30 km long transects to cover both the valley and mountain regions. Samples were analysed for 93 CUPs. In total, 63 different CUPs (29 fungicides, 19 herbicides and 15 insecticides) were detected in all samples (n = 186). CUPs were recorded in 97% of all vegetation and 97% of all topsoil samples (76 of 78 samples each). In total, 140 unique mixtures with ≥2 components were recorded. A prediction map using additional site parameters suggests a widespread presence of CUPs extending multiple hundred meters beyond CUP application areas. Landscape-scale mixture contamination is not addressed in environmental risk assessment for the regulation of pesticides. The study design could serve as a benchmark for evaluating landscape-scale pesticide contamination after implementation of pesticide reduction efforts in agricultural policies and practice.

Pesticides are known to make comprehensive contributions to the decline of several groups of organisms and biodiversity[1–3]. The air in agricultural intense landscapes is particularly loaded with the Current Use Pesticides (CUPs)[4], and CUPs can be transported over long distances[5]. Thus, CUPs have been detected not only in crop fields[6–8] but also in non-target areas[9–13] or even in insects in nature conservation areas[14]. CUPs from agricultural landscapes are able to enter local food webs, potentially magnifying with increasing trophic level[15]. Although a relationship between CUP contamination and short-distance distribution from crop fields has been demonstrated, we just begin to understand CUP contamination at the landscape-scale and the potential exposure of organisms[16].

The complexity of CUP transport processes makes it difficult to predict the exposure of different environmental matrices and non-target organisms, although realistic data on exposure are urgently needed in view of the rapidly advancing biodiversity declines in terrestrial habitats[17,18]. One way to thoroughly evaluate realistic scenarios is to conduct large-scale, long-term monitoring studies to determine the presence of CUPs in the environment. The United Nations Environment Programme calls for additional post-registration monitoring of pesticides and their transformation products in the environment[19], especially in areas where there is high use intensity. Long-term monitoring is already conducted for European surface waters[20], but, in contrast, only a few local and short-term soil and vegetation CUP measurements exist[9,16,21,22], despite the recognized terrestrial biodiversity losses. To assess realistic terrestrial pesticide contamination on a landscape-scale, we conducted extensive sampling across various environmental matrices on a large spatial scale in the Upper Rhine Valley in Central Europe.

The study area is characterized by intensive agriculture, with cropping areas covering 23.4% of the total study region. A diverse range of crops is cultivated, including vegetables (3.4%), cereals (12.2%), vineyards (3.8%) and fruit orchards (2.2%) with some areas exhibiting a high density of cultivated land (Fig. 1, Supplementary Table 1, see methods for details). In the agricultural core of the valley, agriculture and human population are concentrated, where approximately 19.6% of the land is urban and 37.3% is cropping area. The agricultural valley is embraced by the two bordering mountain regions: the Black Forest and Palatinate Forest with low agricultural activity (9.0% cropping area). There are non-target areas of

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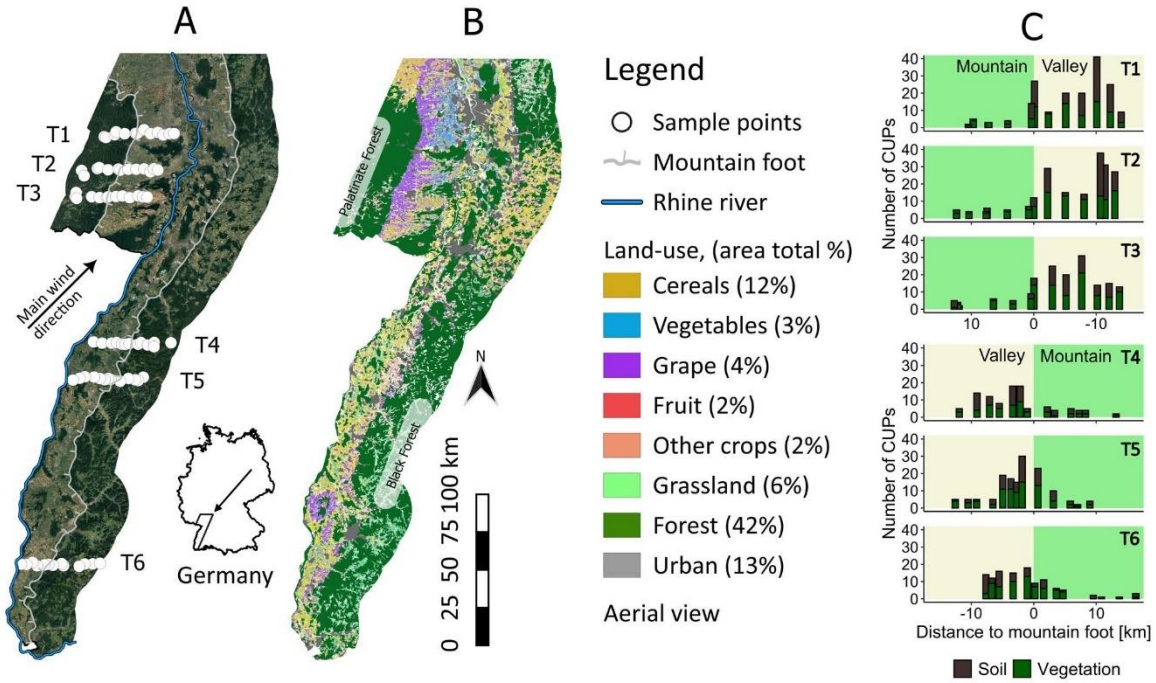


Fig. 1 | Aerial view of the study region Upper Rhine Valley, Germany, its land use and measured cumulative number of Current Use Pesticides residues in topsoil and vegetation per transect. A Aerial view with the 78 sample sites (white dots) and six transects (T1-T6). Sample sites were located on line transects T1 - T6 (north to south) with west/east orientation and categorized into mountain and valley depending their location regarding the mountain foot. **B** Land-use (**C**) Measured cumulative number of CUPs in topsoil and vegetation per transect. Water was excluded in (**C**), due to the availability at only 30 sites. Vegetation and topsoil were sampled between June 15th and July 11th 2022. Digital Elevation Model and forest/urban land cover from European Environment Agency (EEA) (2016); remaining land use categories from Blickensdörfer et al. (2021) of the year 2019. Aerial view: © 2025 Google, TerraMetrics.

conservation concern in the mountain regions as well as in the valley, e.g. nature conservation areas with red-listed plant and animal species. In addition, the Upper Rhine Valley has an alluvial plain and is lying on one of Europe's largest groundwater resources which is under profound pressure due to intensive agriculture[23] and source of drinking water for more than seven million people[24]. Non-target areas outside cropping fields were sampled in the agricultural valley and forested mountain regions utilizing a standardized methodology[9,25], and vegetation, topsoil and surface water were analysed for 93 CUPs. Samples were also taken in the Black Forest National Park, the UNESCO Palatinate Forest-North Vosges Biosphere Reserve and Black Forest Biosphere Reserve.

We assumed that due to the prominent SW wind direction, the Palatinate Forest is less contaminated by CUPs than the Black Forest (Fig. 1) and that CUP residues are detected in higher numbers and concentrations next to agricultural fields and only occasionally further away from the application areas. CUP measurements and land-use data were used to create landscape-scale contamination prediction maps with an area of 10,976 km², that can be used to assess the extent of environmental exposure in non-target areas. We specifically addressed the potential contamination of nature conservation areas in the Upper Rhine Valley.

Results & discussion

CUP residues detected

CUP detections in topsoil, vegetation and surface water. In total, 63 of 93 analysed CUPs consisting of 29 fungicides (F), 19 herbicides (H) and 15 insecticides (I) were detected in topsoil, vegetation and surface water samples from the 78 non-target, off-field sites along the six line transects (Fig. 1A). More specifically, 50 CUPs (24 F, 16 H and 10 I) were detected in topsoil and 43 CUPs (23 F, 8 H and 12 I) were detected in vegetation. In surface water samples taken from rivers, small streams, ponds or puddles, depending on the availability at the non-target sample sites, 44 CUPs (21F, 14 H and 9 I) were detected (Supplementary Table 2). Our total number of 63 CUP detections doubles the recent findings of 27 different CUPs (10 insecticides, 11 fungicides, and 6 herbicides) in the Vinschgau valley in South Tyrol (Italy), which is the largest apple growing area in Europe[16]. One explanation for the higher number of CUPs in the Upper Rhine Valley could be the high crop variability consisting of cereals, vegetables, grape, fruit and other crops (Fig. 1B), requiring different pesticide applications sequences during the season. In addition, the high crop diversity (Supplementary Table 1), as well as the presence of viticulture, could explain the higher number of herbicides and fungicides compared to the Vinschgau Valley. With the Vinschgau Valley's focus on apple growing, pesticide use is both frequent and intensive: on average 38 pesticide applications were conducted from March to September in 2017, with up to 9 different pesticides in one application[26]. The very specific application sequence in apple growing could explain the smaller number of CUPs found in total and different ratios of fungicides, herbicides and insecticides compared to the Rhine Valley. Herbicides have a high sale volume of 48,269 tons (formulation) in Germany, followed by fungicides with 30,072 tonnes (formulation) in 2022[27]. Geographically, Europe is the dominant market for fungicide use in the production of fruits, vegetables, wheat and grape[28], which could explain our frequent detection of fungicides in the Upper Rhine Valley. The most frequently detected CUPs in topsoil in our study were the fungicides fluopyram (94% of all samples), boscalid (42%), spiroxamine (37%), and pyraclostrobin (22%). In vegetation, the most commonly recorded CUPs were the fungicides fluopyram (92%), spiroxamine (55%), cyflufenamid (41%), and boscalid (38%). In surface water, the predominant detections included the fungicide fluopyram (77%), the insecticide pirimicarb (67%), the herbicide metazachlor (63%), and the insecticide tebufenozide (63%). Each of these

frequently detected CUPs is categorized as "High alert" or "Moderate alert" by the Pesticides Properties Database[29] in at least one of the three categories "Environmental fate", "Ecotoxicity" and "Human health" (Supplementary Table 3). Of all 63 detected CUPs, 35% are categorized as "High alert" in "Environmental fate", 43% as "High alert" in "Ecotoxicity" and 40% as "High alert" in "Human health".

The majority, 74% of all 1195 detected individual CUP concentrations, were <5 ng g^{-1} (37 CUPs in topsoil, 21 CUPs in vegetation and 42 CUPs in surface water were only detected with concentrations <5 ng g^{-1}). Nonetheless, even individual CUP concentrations can reach comparatively high values in non-target areas as they were detected for fluopyram and boscalid in topsoil (44.25 ng g^{-1} and 51.86 ng g^{-1} both at T1_7) and vegetation (877.61 ng g^{-1} at T2_6 and 380.57 ng g^{-1} at T5_6), due to the distance of <10 m to the nearest agricultural field (Supplementary Table 4) where pesticide exposure is generally higher[30].

Some of the frequent detections could be related to the sales volume in 2022 and their persistence in the environment: metazachlor (H) had a relatively large volume of 517 metric tons of active ingredient (t a.i.), while spiroxamine (F) had 287 t a.i., pyraclostrobin (F) 151 t a.i. fluopyram (F) 113 t a.i. and boscalid (F) 86 t a.i[27]. Moreover, fluopyram and boscalid are highly persistent substances with a typical DT50 (half-life) in soil of 309 days and 484 days, respectively, resulting in a risk to accumulate[29]. Fluopyram and cyflufenamid are categorized as Per- and Polyfluoroalkyl Substances (PFAS), also known as "forever chemicals", and are part of the 37 authorised PFAS pesticides in the European Union[31]. Persistent and widely occurring substances must be considered critically, especially if there is evidence of effects on organisms. Fluopyram, which was detected nearly everywhere (73 of 78 topsoil samples, 72 of 78 vegetation samples 23 of 30 surface water samples), is used as a fungicide but also registered as a nematicide in tropical crops and shows strong effects on target- and non-target nematode communities in topsoils[32]. Fluopyram also poses ecological risks by inducing behavioural alterations in fish and increasing muscle lipid content, with potential involvement of neurotoxic mechanisms[33]. Additionally, growth anomalies in grapevine, caused by a fluopyram metabolite were reported[34]. Fluopyram was detected in 69% of French soils[10] but only on one site in the apple-growing Vinschgau Valley[16], possibly because it is not recommended for integrated apple production there[35]. In addition, Fluopyram and other PFAS degrade into trifluoroacetate (TFA), a highly persistent and mobile contaminant that threatens water resources[36]. With current chemical assessments failing to address its long-term accumulation, mixture toxicity, and persistent environmental risks[37] we underline the risk of widespread fluopyram presence in the Upper Rhine Valley with its groundwater reservoir.

In the case of boscalid, we detected a contamination of 42% of all sampling sites in topsoils (max. 51.86 ng g^{-1}), 38% (max. 380.75 ng g^{-1}) in vegetation and no contamination in surface water. French soils showed a contamination of 85% of their sampling locations with levels up to 1,211 ng g^{-1} in a cereal field[12], whereas in the Vinschgau Valley boscalid was detected in 11% of the soil samples[16]. The fungicide boscalid can decrease the reproductive quality of honey bee queens and is one of the most common pesticides detected in beehives[38]. In addition, boscalid can induce a mitochondrial dysfunction in human cells, suggesting that it is not selective for fungi targets[39]. The fungicide spiroxamine, detected in more than every second vegetation sample and every third topsoil sample, can increase the mortality of the beneficial parasitical insects *Anagyrus vladimiri* and *Trichogramma evanescens* in viticulture[40], indicating the possible impact of CUP contamination on non-target organisms and the reduction of biological pest control potential. Our results show that there can be regional differences (Rhine Valley vs. Vinschgau Valley) but also similarities in terms of pesticide contamination (Rhine Valley vs. France), which results in a need of individual environmental pesticide exposure evaluation. Although the EU National Action Plans ask for the implementation of integrated pest management, which should be adapted to local conditions and crop types, this strategy has not been effective since pesticide use has not decreased in the EU so far[41,42].

CUP residue mixtures in topsoil, vegetation and surface water. CUPs were detected in 97% of all vegetation and 97% of all topsoil samples (76 of 78 samples each). Surface water was available for sampling at 30 of the 78 sample sites, and CUPs were detected in 83% of the samples (25 of 30). Of the total 63 detected CUPs, 27 CUPs (43%) were recorded in all three matrices (vegetation, topsoil and water), 20 CUPs (32%) were detected in at least two of the three matrices and 16 CUPs (25%) were detected in only one of the three matrices (Supplementary Table 5). The detections in almost all combinations of the three matrices and the spatially distributed sample sites with potentially specific habitat conditions, ranging from intensive agriculture in the valley to remote locations in the mountains, show a high probability of pesticide exposure at both patch (site) and landscape (transect) scales (Fig. 2). In total, 140 unique mixtures with ≥2 components were recorded. Of the soil, vegetation and surface water samples, 76%, 87% and 80% contained mixtures, respectively. Samples from non-target, off-field habitats in the agricultural valley showed 90 unique mixtures, whereas 54 unique mixtures were recorded in forested mountain regions. The most often detected mixture contained the fungicides fluopyram + spiroxamine, but this specific mixture occurred only 6 times, indicating the high variability of mixture composition. All other mixtures occurred once (95.7%), twice (2.9%) or threefold (0.7%), demonstrating that there was site-specific contamination. We detected a maximum of 26 CUPs in topsoil, 21 in vegetation and 39 in surface water samples in the agricultural valley (T1_3, T3_4 and T1_3, respectively). Fungicide-intensive viticulture on slopes bordering mixed crops with vegetables, fruit orchards and cereals is typical of this area in Southern Palatinate. Sites without CUP residues in vegetation, soil or surface water were measured only in remote mountain regions (T1, T2, T6 mountain sites 8–13, Fig. 2). However, there was no site where all matrices were uncontaminated. This is in line with a study from the Italian alps, where none of the non-target sites were without CUP detections in soil and vegetation[16]. This CUP exposure could initially have a direct effect on non-target organisms, in case of herbicides directly affecting non-target plants outside of cultivated areas. In addition, herbicides can affect not only plants but various other non-target organisms including humans[43]. Cases of direct acute bee mortality and sub-lethal impairment of their normal biological behaviour have been documented for herbicides, and fungicides and synergistic effects when in mixture with other agricultural chemicals[44]. Traces of insecticides threaten numerous non-target organisms by affecting traits of the individual, including development, physiology, behaviour and communication[45]. In a recent study involving over 1000 pesticides, exposure to sublethal doses (reflecting typical field application and low environmental concentrations) in *Drosophila melanogaster* revealed that 57% of the studied pesticides significantly altered larval behaviour after short-term exposure[46]. Key traits, such as egg-laying rates, reducing reproduction and in the end leading to population decline, were reduced by some pesticides even at concentrations several orders of magnitude below sublethal levels[46]. In addition, nonlinear interactions between many combinations of highly prevalent pesticides suggested widespread synergistic interactions[46]. It can therefore be expected that sublethal effects can also occur at low environmental concentrations, similar to those measured in this study. Our detected high variability of mixtures indicates the difficulty of generalizing CUP exposure and resulting potential effects. The risk of environmentally occurring CUP mixtures is not assessed for the regulation of pesticides in the EU, where only single substances or a few pesticides that are prepared in a single formulation are addressed. CUPs are only tested in the laboratory and in field experiments with a small number of model organisms (Commission Regulation (EU) No 284/2013, 2013) and unexpected consequences due to interactions of different substances (e.g. additive or synergistic effects) or sublethal effects and indirect effects on other organisms are not investigated[47–49]. A realistic assessment of CUP mixture exposure and its associated risks to biodiversity would be extremely challenging, given a large number of different mixtures recorded. The mean number of CUP detections differed between area and

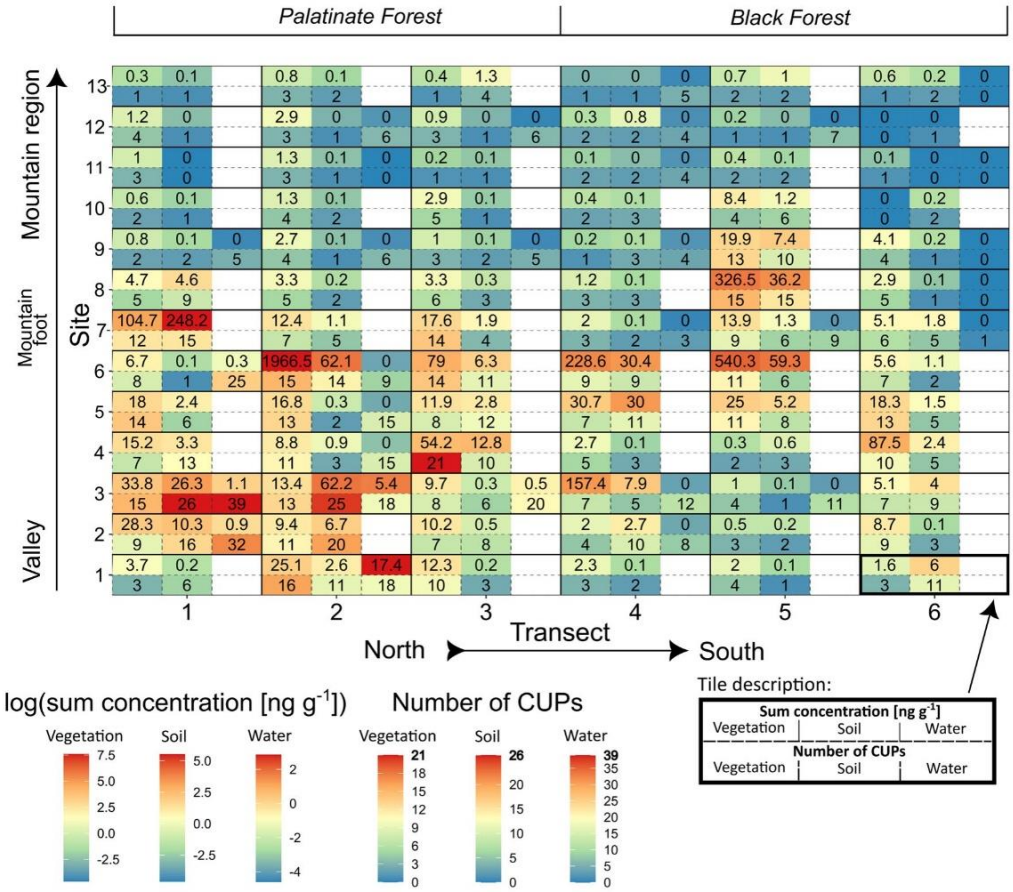


Fig. 2 | Detected Current Use Pesticides sum concentrations and number of residues. Explicit results of the 78 sampling sites for the number and sum concentration of CUPs. Each of the six line transects consisted of 13 sample sites. In each tile, vegetation (bottom left), topsoil (bottom middle) and water (bottom right) and their corresponding sum concentration in ng g^{-1} in the cells above is mentioned (see tile description). Regions defined as valley and mountain region, Palatinate Forest and Black Forest and North and South. Site 1 was always closest to the Rhine in the valley, site 7 at the mountain foot and site 13 most remote in the mountain region. For details on the location of the transects see Fig. 1.

matrices: On average, 6.3 CUPs were found in vegetation (9.2 in the valley and 3.2 in mountain regions), 5.4 CUPs were found in topsoil (8.2 in the valley and 2.3 in mountain regions) and 9.6 CUPs were found in surface water (16.7 in the valley and 3.3 in mountain regions). An analysis of the European soil database, focusing on 76 target CUPs, with an analytical sensitivity of >10 ng g^{-1} (LOQ) for LC-MS/MS compounds and >5 ng g^{-1} (LOQ) for GC-HRMS compounds, showed a mean of 2.7 (median = 3) pesticides in European agricultural soils and 2.4 (median = 1) in Germany[8]. Their mean number of detected CUPs in non-target area topsoils is up to two times lower, compared to our measurements with 93 target CUPs. We included measurements in the number of CUP detections when they reached individual LOD levels and included measurements in site sum concentrations when individual LOQ levels, up to 1000 times lower (topsoil LOQ: 19.11–0.008 ng g^{-1}, vegetation LOQ: 21.5–0.01 ng g^{-1}, water LOQ: 0.01–0.0006 ng g^{-1}, Supplementary Table 6) compared to the previously mentioned study, were reached. Differences in extraction, analytical methods as well as detection limits and number of target CUPs make comparisons difficult, however the analytical sensitivity of our study showed a higher CUP number in non-target areas compared to the target in-field samples of the EU study. Additionally, the EU study was an in-field measurement whereas we sampled in the non-target off-field area. Since 281 different CUPs are currently authorised in Germany[27] and 440 in Europe[50], the number of CUPs and sum concentration is even higher than our results show as only 93 CUPS were included in our analysis. In 2021, a study with the same analytical method was conducted close to the transects T1, T2, T3 but also with samples from cropping areas[22]. The results show that the number of CUPs decreases with distance to the target site but crop type and season play a critical role in contamination prediction[11,22]. It can therefore be concluded that there is an even higher number and sum concentration of CUPs present in fields, vineyards and orchards close to our non-target areas.

We demonstrate a widespread contamination with CUP residues across different environmental matrices and a high variability in mixture compositions. The presence of unique and site-specific mixtures underscores the complexity of CUP exposure in non-target areas, raising concerns about unassessed ecological impacts from diverse, co-occurring residues.

CUP sum concentration in topsoil. Summing up the concentrations of recorded individual CUPs, we detected a mean CUP sum concentration of 8.48 ng g^{-1} in all topsoils, ranging from no detections to a maximum value of 248.23 ng g^{-1} (T1_7, <10 m to vineyards). This is in the same CUP sum concentration range as recently measured in topsoils of the Vinschgau Valley with a mean of 4.15 ng g^{-1} [16]. When split into valley and mountain regions, the valley topsoil showed a 26-fold higher mean concentration (15.97 ng g^{-1}) in comparison to mountain region topsoil

(0.60 ng g^{-1}). A recent study in France revealed 0.1 to 76 ng g^{-1} with a mean value of 30.4 ng g^{-1} in French grassland topsoils and <2 ng g^{-1} in forest topsoils (only >LOQ, 110 target substances)[10] which is comparable to our detections in valley and mountain regions, respectively. A comparison of sum concentrations is difficult due to the lack of comparative studies and the dependence of the method and detection limits (see above). A 15-fold higher mean was detected in French grassland compared to French forest topsoil[10] which is also comparable to our valley (26 times higher) versus mountain region detections. The top ten topsoil CUP sum concentrations (248.23 ng g^{-1} to 10.34 ng g^{-1}) were measured in the valley (Supplementary Table 4).

CUP sum concentration in vegetation. In vegetation, a mean value of 51.21 ng g^{-1} was detected (max: 1966.51 ng g^{-1} at T2_6, <10 m to vineyards), which is slightly lower than the recorded mean CUP sum concentration of 64.5 ng g^{-1} in playground grass and 104.48 ng g^{-1} in vegetation in valleys in South Tyrol[16,51] The study area in these studies is dominated by apple orchards in the valley whereas in our case vineyards are concentrated at the mountain foot and fruit cultivation is more diffuse in the Upper Rhine Valley. One exception is the fruit cultivation area at the mountain foot close to T4_6 and T5_6, possibly explaining the higher sum concentration detected there (Fig. 2). Another explanation for our slightly lower mean sum concentration in vegetation could be the large number of sites with greater distance to the next agricultural activity (mean distance of 650 m). The vegetation of the valley in the whole study area showed a 40-fold higher mean CUP sum concentration (97.55 ng g^{-1}) in comparison to mountain region vegetation (2.43 ng g^{-1}). The top ten vegetation sum concentrations (1,966.51 ng g^{-1} to 33.85 ng g^{-1}) were measured in the valley (Supplementary Table 4). The highest concentration of 1,966.51 ng g^{-1} was mainly driven by the fungicides fluopyram and tebuconazole with 877.61 ng g^{-1} and 777.46 ng g^{-1} respectively, detected together with 13 other CUPs at this sample site (T2_6 distance of <10 m to the nearest vineyard), making contamination by drift from viticulture likely. Three-dimensional application in viticulture results in greater pesticide drift compared to arable applications and greater exposure of near non-target areas[22,30].

CUP sum concentration in surface water. In surface water, a mean CUP sum concentration of 0.86 ng ml^{-1} (max: 17.41 ng ml^{-1} at T 2_1, puddle <10 m to the next field) was detected and the top ten maximum water sum concentrations (17.41 ng ml^{-1} to 0.01 ng ml^{-1}) were all measured in the valley (T1-4). Of the 30 surface water samples (Supplementary Table 7), six were taken from puddles with the highest measured sum concentrations (top six) of 17.41 ng ml^{-1} to 0.30 ng ml^{-1} and 39 to 18 different CUP detections. It can be assumed that the CUPs are concentrated by desiccation, which turns puddles in agricultural areas into toxic soups. Puddles in human modified landscapes are a an important drinking water source for birds[52] and honey bees actively forage in "dirty" water sources for minerals that may be lacking in their floral diet[53]. We emphasize the critical exposure pathway for birds and mammals as well as other vertebrates and invertebrates through water uptake, especially when contaminated puddles are the only source of water in hot seasons or during increasingly frequent droughts. Realistic puddle exposure needs to be addressed as a critical source for affecting biodiversity, and in-field puddle exposure is expected to be even higher. The 19 sampled small streams showed sum concentrations up to 0.03 ng ml^{-1} (Supplementary Table 7) whereby small streams in the valley as well as in the mountain regions were contaminated (15 of total 19 small streams). A study of 124 monitoring stream sections across Germany found that pesticides in agricultural streams were the dominant driver in reducing vulnerable insect populations in aquatic invertebrate communities and event-driven sampling was used to additionally record surface rainfall-induced short-term peak concentrations[54]. In addition to effects in the aquatic environment, terrestrial food webs in the riparian zone may be impacted via reduced insect emergence[55]. The neonicotinoids acetamiprid and thiacloprid, detected in two small streams (T4_3 and T5_7), were the most frequently detected pesticides in 90–100% of emerging insect samples in a study in the same region and were shown to biomagnify in riparian spiders[25]. We just begin to understand the complexity of the aquatic-terrestrial meta-ecosystem[55] and CUP contamination studies which integrate both, aquatic and terrestrial matrices, could support biodiversity conservation efforts by indicating relevant effect pathways. Water sources are quite diverse, and substance transport can vary significantly between small streams and other sources, such as ponds or puddles.

CUP residue gradient and wind effects. Across all matrices, a higher number of CUPs and sum concentration was detected in the valley (mean number of CUPS = 9.6 CUPS, mean sum concentration = 46.7 ng g^{-1}) compared to the mountain region (mean number of CUPS = 2.9 CUPS, mean sum concentration = 1.4 ng g^{-1}. Matrix specific, there was a significant higher number of CUPs in valley topsoil ($z_{76} = 10.51$, $p < 0.001$), vegetation ($z_{76} = 10.08$, $p < 0.001$) and surface water ($z_{28} = 10.64$, $p < 0.001$) as well as a higher sum concentration in valley topsoil ($t_{76} = 6.18$, $p < 0.001$), vegetation ($t_{76} = 7.03$, $p < 0.001$) and surface water ($t_{28} = 3.95$, $p < 0.001$, Supplementary Table 8). The number of CUPs in vegetation and topsoil decreased from the valley to the mountain regions, only increasing by approximately two more CUPs at the mountain foot (Fig. 3A). Despite the decreasing CUP numbers a tenfold increase in the CUP sum concentration in vegetation and topsoil could be observed at the mountain foot compared to the valley (Fig. 3B). This suggests that a detailed representation of landscape contamination can only be obtained with both values. Our results indicate, that CUPs can travel multiple hundred metres from the agricultural valley into forested mountain regions and that the contamination is related to the distance to the mountain foot in both directions. As the mountain foot of the Upper Rhine Valley is widely covered with vineyards and interspersed with fruit cultivation, the observed increase in CUP numbers and especially sum concentrations could be related to the 3-dimensional application required in both crop types and resulting drift. In addition, vineyard site conditions like a high air temperature and wind velocity, enhance pesticide volatilization from target plants and soils[56] which could favour atmospheric transfer.

To test the effect of the main wind direction on the number of CUPs in mountain regions, a Welch Two Sample t-test was applied for all CUP detection numbers in soil and vegetation in mountain areas ($n = 38$). With a main wind direction from southwest to northeast, the mountain areas in T1, T2, T3 would be contaminated upwind and T4, T5 and T6 would be affected downwind, if a connection between wind and CUP transport is assumed. No significant effect could be found for the number of CUPs in vegetation ($t_{32.63} = 0.47$, p-value = 0.640) and the sum concentration in vegetation ($t_{33.86} = -0.411$, p-value = 0.683). However, there was an average trend of 1.51 more CUPs ($t_{30.20} = -1.79$, p-value = 0.084) and 0.699 ng g^{-1} higher sum concentration ($t_{22.14} = -1.72$, p-value = 0.099) in topsoil of the Black Forest mountain regions (T4, T5, T6) which would be contaminated with the main wind direction. Wind can have a direct influence on the direction, transport distance and concentration of pesticides as well as on the transported droplets deposition place[57]. The fact that pesticides are found in ambient air underlines this possible distribution path[4,58,59]. The number of pesticides in air was shown to increase with higher air temperature and decrease with less precipitation[4]. Pesticides have been found in rainwater[60], demonstrating just another aspect in complex meteorological systems accountable for non-target site contamination. Semi-volatile pesticides can get adsorbed on atmospheric aerosol particles which leads to high persistence and long-range transport (>1000 km) in the atmosphere[61]. Nonetheless, it has been shown that long-range CUPs come from different chemical classes with very different physicochemical properties, resulting in the assumption that the assessment of these properties cannot fully explain their distribution[62]. The occurrence of CUPs in remote mountain regions of the Upper Rhine Valley through medium-range transport (1–1000 km)

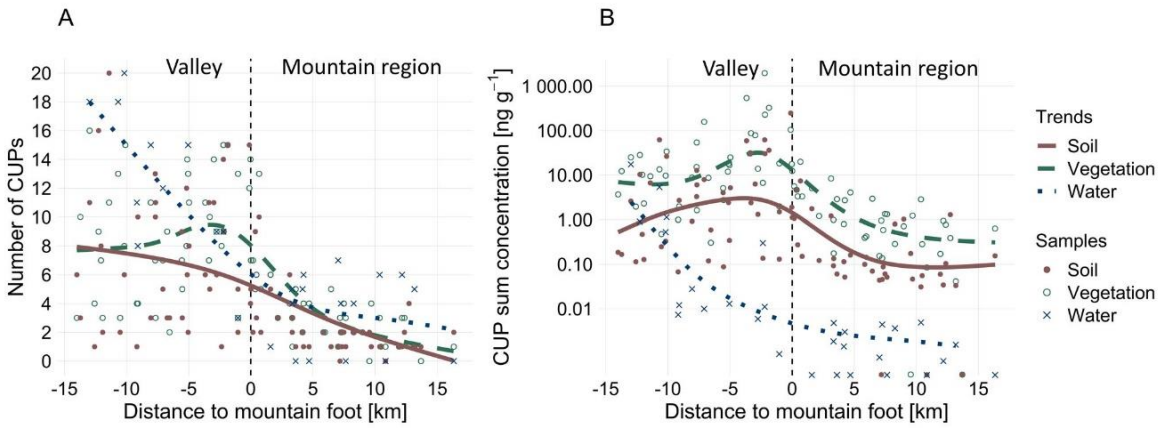


Fig. 3 | Gradient of detected Current Use Pesticides residues from valley to mountain regions. A Number of detected CUPs in vegetation (circles), topsoil (dot) and surface water (cross). **B** CUP sum concentrations in ng g^{-1} in vegetation, topsoil and surface water. Loess smooth trendlines indicate the gradient from the valley (-15 km to 0 km) to the mountain regions (0 km to 15 km). 0 corresponds to the foot of the mountain that defines the Rhine Valley by sudden altitude and land use changes.

questions current pesticide regulation. Medium-range transport, which results in landscape scale CUP contamination, is hardly addressed in current regulation where the main concern lies on short-range transport (drift) and long-range transport, for example to Arctic regions[5].

There is also some agricultural activity in the remote mountain regions which could contribute to CUP occurrence (Supplementary Table 1). The maximum distance from a transect site to agricultural cropping areas (including cereals, fruits, vegetables, grape, other crops) was 4.4 km (T1_10). This site showed contamination of fluopyram and spiroxamine in vegetation and fluopyram in soil. The contamination in the mountain regions with 2–3 pesticides (calculated mean over all matrices = 2.9) can be considered as a ubiquitously present background contamination. As mentioned before, this number will be even higher in reality, as not all approved CUPs were included in our analytical procedure. In summary, pesticides in the Rhine Valley occur not only in the immediate agricultural landscape in the valley itself, but also with a decreasing gradient in the surrounding mountain regions, which are therefore not exempt from CUP contamination, although their sensitive ecosystems could be less adapted to agricultural influence.

Prediction. With the categorized and spatially weighed land use data, the number of CUPs and sum concentrations of off-field topsoil and vegetation during the spraying season was predicted for the area of the Upper Rhine Valley with a resolution of 1 × 1 km (see Supplementary Information for further details). The generalized linear model with a Poisson distribution for the number of CUPs in topsoil and vegetation had multiple R^2 values of 69% and 76%, respectively. The linear model with normal distribution for the sum concentration of CUPs in topsoil and vegetation achieved good explanatory power with multiple R^2 of 62% and 71%, respectively. The model components reveal fruit, grape and vegetables as driving factors for a higher number of CUPs in vegetation and a sum concentration of CUPs in topsoil and vegetation (Supplementary Table 9, 11, 12). A higher number of CUPs in topsoil was driven by vegetables and other (not further categorized) crops (see methods and Supplementary Table 10). Overall, land use types result in the exposure of the surrounding land much further away than expected using drift extrapolations[30], often for multiple hundred metres (See methods section for further details, Supplementary Fig. 1–4). A mean exposure of 4.6 CUPs was predicted for topsoil and 4.3 for vegetation with a maximum of 27 for topsoil and 32 for vegetation (Supplementary Table 14). Contamination in the non-target, off-field areas differ depending on location and neighbouring crops, which can be seen in the varying patterns of the generated maps (Fig. 4).

According to the resulting prediction map, non-target areas located in the agricultural vicinity, are exposed to multiple CUPs in complex mixtures and high sum concentrations, especially in the valley. In the valley and mountain regions of the study area, 37.3% and 9.0% of the land is used for agriculture, respectively (cropping areas without grassland, Supplementary Table 1). A hotspot for the number of CUPs in vegetation was predicted for the slopes at the mountain foot in the southern Palatinate (T1, T2, T3) or the Kaiserstuhl (north of T6), both of which are areas with intense viticulture (Fig. 4, land use). In Europe, the majority of pesticide products for specialty crops are used in viticulture[63]. Approximately 7.2% of the land in the valley (without the mountain region) is used for viticulture but 18.4% for cereals, 6.4% for vegetables, and 2.7% for fruit production[64] (Supplementary Table 1). A slightly different pattern is predicted for topsoil with maxima in a number of CUP detections for the slopes in southern Palatinate (T1-3), too, but also at the valley floor, in the north-western part of the study area (T1). In this region of the valley, vegetables are grown in a large area which contributes to a high predicted number of CUPs. Both topsoil and vegetation show differentiated hotspots within a fruit-growing area close to Achern also called the "Baden fruit paradise"[65] and the wine-growing area Kaiserstuhl. In terms of the amount of pesticide product usage ranks, vegetables and fruit trees follow after viticulture[63]. Predicted sum concentrations reflect an increase in the contamination of vegetation on the wine-growing slopes as shown by the measurement results. This also applies partly to the predicted sum concentration in topsoil, although individual hotspots are more narrowly defined here. The contamination of vegetation appears to be influenced by land use over a greater distance than the contamination of topsoil, as can be seen from the greater distance of explaining land use in the prediction model (Supplementary Table 13).

However, CUP contamination is not limited to agricultural hotspots but is predicted area-wide to occur in the Upper Rhine Valley: more than one CUP is predicted in 98% of topsoil and vegetation samples and contamination-free sites are only expected in remote mountain regions, larger forest blocks or in centres of larger cities that are not closely surrounded by agriculture. We emphasize that this prediction map only shows CUP estimations for non-target areas during the spraying season. A complete contamination map including the target-areas, the cropping areas, is expected to show higher values of CUP numbers and sum concentrations over large areas.

Nature conservation areas. For nature conservation areas (NCA, not including national parks or biosphere reserves) inside the study area, contamination was predicted with a resolution of 200 x 200 m. NCAs are among the most strictly protected areas in Germany[66]. A mean of 4–5

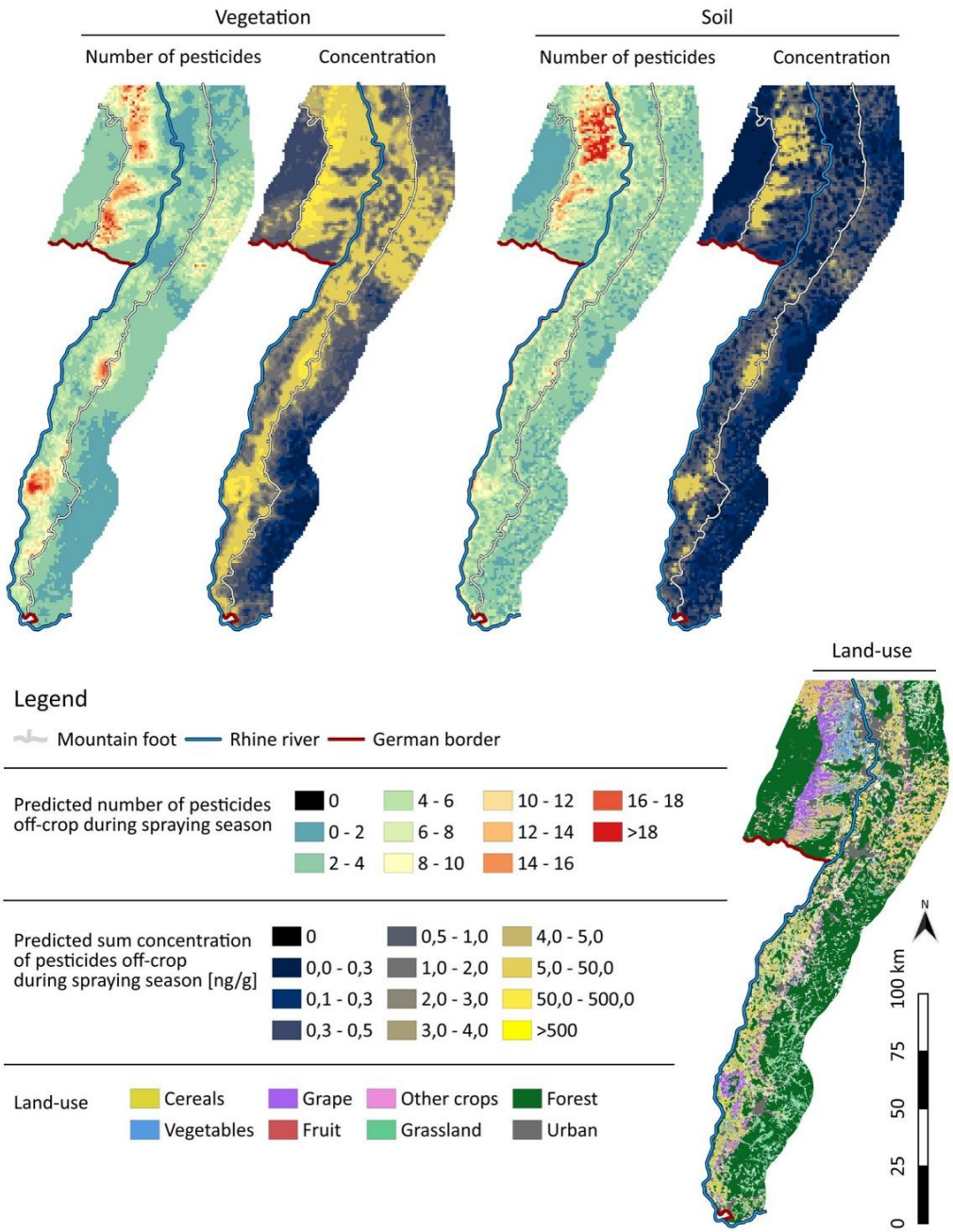


Fig. 4 | Predicted Current Use Pesticides sum concentrations and number of residues in off-field vegetation and topsoil during spraying season. The number of CUPs and sum concentration ng g^{-1} in off-field vegetation and topsoil during spraying season were predicted with land use categories in a linear model (CUP sum concentration) or generalised linear model (CUP number). Multiple R^2 is between 0.62 and 0.76. The raster pixel size is 1 × 1 km. Forest/urban land cover data are from the European Environment Agency (EEA) (2016); the remaining land use categories are from Blickensdörfer et al. (2021). The prediction map is based on the topsoil and vegetation measurements and their 78 sites only.

CUPs was predicted in their vegetation and 5–6 CUPs in topsoil (Supplementary Table 15), which shows that NCAs are not exempt from CUP exposure and are not necessarily refuge areas for protected plants and animal species. Species richness of butterflies decreased in NCAs and other protected areas in Germany[67] as well as insect biomass by more than 75% over 27 years[17]. Insects in NCAs bordering agricultural areas have been shown to be directly exposed to pesticides[14]. In our extrapolation some areas are predicted to be at particular risk due to their location: A maximum value of 19-20 CUPs is predicted for the vegetation of the "Badberg" on the Kaiserstuhl, which could be explained by the intensive surrounding viticulture. The Kaiserstuhl inhabits 16 different NCAs. Non-target and structurally rich margins in agricultural landscapes have been shown to be important for rare and red-listed species[68] and habitats available for non-target organisms in general and terrestrial biodiversity.

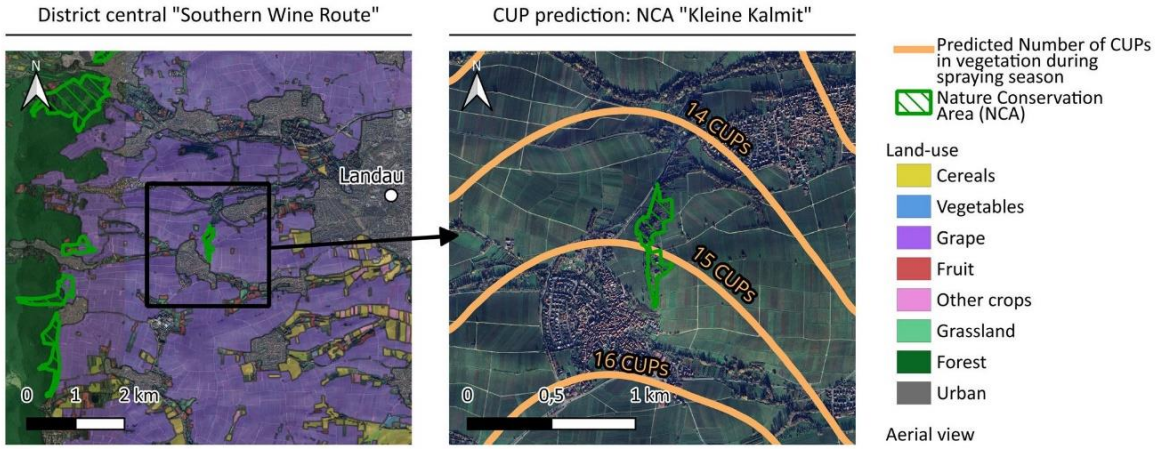


Fig. 5 | Predicted number of Current Use Pesticides residues in the non-target Nature Conservation Area "Kleine Kalmit", Southern Palatinate, Germany. The number of CUPs in off-field vegetation during the spraying season was predicted with a resolution of 200 × 200 m. Multiple R^2 of the glm generated with measurements of the number of CUPs in the overall study area = 0.76. Forest/urban land cover data are from the European Environment Agency (EEA) (2016); the remaining land use categories are from from Blickensdörfer et al. (2021). Aerial view: © 2025 Google, TerraMetrics.

Therefore, one of the main objectives of the Common Agricultural Policy (CAP) of the European Commission is maintaining and increasing landscape features such as hedges, field margins, dry-stone walls and isolated trees to foster biodiversity in agricultural landscapes[69]. The potential of such habitats remains to be critically questioned if they cannot be kept pesticide-free. Our results suggest that the compensation potential of non-target areas established or maintained explicitly for biodiversity in agricultural landscapes strongly correlates with the distance to CUP target areas – the cropping area. This is underlined by the prediction of a maximum of 19–20 CUPs in topsoil for the "Sandgrube bei Schauernheim", a relatively small NCA of 12 hectares surrounded by vast agricultural activities. A nature conservation area in an agricultural neighbourhood with a small size and associated small buffer zone can therefore simply induce high CUP pressure[14]. Another representative example is the "Kleine Kalmit" (5.7 ha), which is situated on a small hill in a large wine-growing area in southern Palatinate (T3). It was predicted to contain a mean of 11–12 different CUPs in topsoil and 14–15 in vegetation during the spraying season (Fig. 5). In another study, 4 CUPs in topsoil and 16 CUPs were detected in vegetation in July 2021 (August: 7 in topsoil and 12 vegetation) in the southern part of the NCA with the same analytical method[22]. Our site T3_6 was closest to the "Kleine Kalmit" with a distance of 1,3 km and 11 different CUPs in topsoil and 14 in vegetation could be detected there. This demonstrates the applicability of the NCA prediction, but particular uncertainties exist due to factors such as the climatic conditions in the sampling year or the soil type at the explicit sampling location.

Our direct measurements in the Black Forest National Park revealed a total of 4 different CUPs. Additionally, at the Feldberg NCA (sampled at 1277 m a.s.l.), 3 different CUPs were detected. Similarly, 3 different CUPs have been shown to reach remote alpine meadows at 2318 m altitude in the Vinschgau Valley[16]. The widespread presence of CUPs in NCAs highlights a critical concern: there is an urgent need to establish larger buffer zones around nature conservation areas[14] and stop the use of substances that can be found in remote areas, such as the fungicides fluopyram, boscalid and spiroxamine. Additionally, pesticide use is not directly prohibited in NCAs in Germany[70] and a general law is needed at the federal level.

Conclusion

Our study reveals that during the spraying season, non-target areas in the agricultural valley and adjacent mountain regions are subject to complex CUP contamination, with varying mixtures depending on the location and surrounding land use. At all study sites there was at least one matrix contaminated, aligning with other studies which find non-target areas contaminated[8,10,16]. Pesticide drift and run-off events from the terrestrial area of landscapes also affects aquatic systems such as small and medium-sized streams, and aquatic contamination again has feedbacks on the riparian terrestrial zone[71]. Natural puddles in non-target areas contained the highest contamination levels and are so far not included in environmental risk assessment procedures. The pervasive spread of CUPs suggests an urgent need to prioritize areas at particular risk of contamination: Our spatial assessments revealed that land use types like fruit orchards, vineyards or vegetable fields affect the surrounding land, often for multiple hundred metres. We underscore the challenges in comparing measurement results, as the number of target CUPs, detection limits and quantification limits still have a significant influence on the detection rate. Nevertheless, we demonstrated the utility of predictive models based on real-world samples to determine background contamination and realistic concentrations, including trace levels of CUPs. It is crucial to quickly anticipate potential environmental stressors rather than relying solely on retrospective data collection, due to the time pressure of the ongoing rapid decline in biodiversity. Our prediction maps might also provide helpful tools in biodiversity management implementation for selecting low contamination areas. CUP measurements and predictions highlight the urgent need to reduce the overall number and amount of substances to (re)establish uncontaminated areas that are crucial for nature conservation and human health. CUP mixtures can reduce the diversity of soil fauna[72] and therefore very likely impact soil fertility, which was recently considered to be directly related to human health[73]. In addition, there are higher levels of pesticide exposure among residents living in close proximity to agricultural lands than among those living at greater distances[74]. Many CUPs are still used preventive[75], but their impact extends beyond fields of application, leading to long-term consequences for biodiversity[1,76]. We show that complex CUP mixtures and their unpredictable components expose entire landscapes and the environmental risk is not assessed correctly. The complex combinations of CUP mixtures and the numerous potential interactions among their components create disregarded environmental pressures across entire landscapes. As this exposure is expected to be chronic as shown for non-target areas in the proximity of 20 m of agricultural fields[22], the current pesticide authorisation and risk management practices do not protect terrestrial biodiversity.

The results of this study highlight the need for changes in agricultural practices in the Upper Rhine Valley, but also for other Central European regions with pesticide-intensive agricultural production to reach global

pesticide reduction targets[77]. More sustainable approaches like the production of baking cereals without the use of synthetic pesticides are possible and already in place for many years[78] and would affect a large proportion of the agricultural landscape in the EU as one-third of the total utilisable agricultural area is used for cereal production[79]. Together with the aim of transforming 25% of the agricultural land to organic production by 2030, as formulated originally in the EU Green Deal[80] and integrated in national and international policies[77,81], synthetic pesticide use could be substantially lowered leading to a reduction of pesticide exposure of biodiversity and humans. Our prediction mapping approach could be repeated with measurements in 2030 to evaluate these reduction efforts.

Methods

Study region

The Upper Rhine Valley ranges from Basel in Switzerland to Bingen in Germany (270 km), embraced by the mountain plateaus of the Vosges Mountains and Black Forest in the south and the Palatinate Forest and Odenwald in the north. The Upper Rhine Valley has a mean air temperature of 8.6 °C, mean annual precipitation of 73.5 cm and 15 large cities with more than 100,000 people[82]. Agriculture is a dominant land use in the valley with vegetables and arable crops in the valley lowland and viticulture being mainly present on the mountain foot. The mountain plateaus are separated from the valley by a sudden rise in altitude – slopes (study sites covered 99 to 1378 m) - and are dominated by forest cover. Data were collected close to Ludwigshafen (north, T1) to Staufen im Breisgau (south, T6). The prediction was limited to the area from Worms to Basel (10,976 km² prediction area) within Germany, which includes part of the Valley, the Black Forest, and the Palatinate Forest. The study area contained parts of the UNESCO Palatinate Forest-North Vosges Biosphere Reserve and Black Forest Biosphere Reserve as part of the Natura 2000 Network, Nature Conservation Areas and the Black Forest National Park.

Sampling and pesticide analysis

Six line transects with west/east orientation and 30 km length were selected to cover the valley as well as the mountain regions (Fig. 1). Thereby, three transects were set in the northern part at the section of the Palatinate Forest (T1, T2, T3), two transects at the section of the Black Forest National Park (T4, T5) and one transect at the section of the Feldberg (1277 m a.s.l.), the highest mountain in the Black Forest (T6). With a main wind direction from south-west to north-east, mountain areas in T1, T2, T3 would get contaminated against and T4, T5 and T6 with the main wind direction if a connection between wind and long-distance transport is assumed. For each 30 km long line transect, six sampling sites were placed in the valley (TX_1 – TX_6), one close to the mountain foot (TX_7) and six in the mountain area (TX_8–TX_13), resulting in 13 sampling sites per transect. The site selection process yielded an average transect length of 31.5 km and an average distance of 2.6 km between sampling sites along the transects. The sites in the mountain area were selected to capture the sequence of the geographic altitude (sequence of mountain and small valley). For CUP area comparison, the 13 sites of each of the six transects were split into valley and mountain region depending on their position regarding the sudden increase in altitude at the mountain foot (sites close to the mountain foot were categorized, too), resulting in a total of 78 sampling sites. Between June 15th and July 11th 2022, composite topsoil samples consisting of 25 subsamples were taken from a grassland area of 4 x 4 m per sample point with a topsoil corer (diameter: 13 mm, 5 cm deep, Rasengrün, Ingelheim, Germany) in a depth of 0–5 cm. Similarly, vegetation representative of the sample site was manually handpicked in the same area and placed in a 1 L plastic bag. The vegetation samples then included a mixture of different parts (excluding roots) of grass, herbs and leaves of shrubs depending on availability. If a surface water source (puddle, stream or river) was present in the immediate proximity of the sample point, a 250 ml water sample was taken. Transportation to the laboratory and storage in a freezer at −20 °C took place after sampling as soon as possible. Vegetation, topsoil and surface water samples were then further processed in the laboratory.

Vegetation and topsoil samples were analysed[9] for 93 CUPs using a high-performance liquid chromatography coupled with an electrospray ionization tandem mass spectrometry system (HPLC-ESI-MS/MS; HPLC: Agilent Technologies LC 1260 Infinity II series, MS/MS: Agilent Technologies 6495C, Santa Clara CA, USA). Surface water samples were analysed for 75 CUPs with a direct-injection HPLC-ESI-MS/MS method[25]. Details on the extraction procedures and analytical HPLC-MS/MS method and instrument parameters can be found in the Supplementary Information.

CUP sum concentrations were calculated for each sampling site as the sum of all single CUP detections above the limit of quantification (>LOQ) in vegetation, topsoil and water separately. Limits of quantification and detection can be seen in Supplementary Table 6.

Land use and topography data

National-scale crop type maps for Germany from combined time series of Sentinel-1, Sentinel-2 and Landsat 8 data (2017, 2018 and 2019) were used as agricultural land use data[64]. Google Satellite Imagery WMS data was used for satellite images[83]. The land use types were grouped into categories "Grassland", "Cereals", "Vegetables", "Grape" and "Fruit". Remaining land use types were grouped into "Other crops", also containing areas specified as "Other agricultural areas". Forest and urban cover was assessed with the Corine Land Cover (CLC) dataset[84] resulting in a total of 8 different land use categories. Further topographic indices (Altitude, Roughness Index, Topographic Wetness Index, Topographic Position Index, Wind Shelter Index and Wind Exposition Index) and environmental factors (Mean land surface temperature for the years 2018–2020) were evaluated for their correlation with land use data by using them in a separate principal component analysis. Due to a strong correlation with agricultural activities, they were excluded for predictions (Supplementary Information for details).

Optimization and spatial weighing of land use categories

We found CUPs far from the agricultural valley, contrary to the expectation of particularly remote and clean mountain regions. We, therefore, asked how far away the agricultural areas are responsible for the occurrence of CUPs in remote regions. To do so, we grouped the land use into 8 different categories ("Grassland", "Cereals", "Vegetables", "Grape", "Fruit", "Other crops", "Forest" and "Urban"), assuming that similar land use types would lead to similar CUP applications. We dissected the categorized land use area into a raster of 10 x 10 m.

We then computed how much of the land with a certain distance σ to a test-site is from each given land-type use. We assumed that areas closer to the sampling site would contribute more to the CUPs detected at the sampling site and therefore we used a (two-dimensional) Gaussian kernel based on distance to the sampling site as weights for the raster points. Specifically, we computed the mean land coverage of type L around a given sampling site i as

$$W_{iL}(\sigma) = \frac{A}{2\pi\sigma^2}\sum_{l\in L}\exp\left(-\frac{(x_i - x_l)^2}{2\sigma^2}\right)$$

where A is the area of a pixel, in our case $10m \times 10m = 100m^2$, σ is the kernel width indicating how much of the surrounding land contributes to the land coverage, x_i are the coordinates of the sampling location and x_l are the coordinates of the raster point. The sum is taken over all areas l with land use type L. The factor in front of the Gaussian kernel ensures that $W_{iL} = 1$ if all the surrounding land would be of type L. The area within 1.96σ of the sampling site contributes to 95% of the weighted area. We computed this mean land type coverage for all 78 sampling sites i, each of the 8 land use types L and for σ varying from 50 to 5000 in steps of 50.

Given these mean land coverages for the sampling sites and land use types we searched for the optimal kernel width σ_L for each land type which would lead to the "best" prediction. Specifically, we fit a generalized linear model (glm) for the CUP richness and a linear model (lm) for the sum

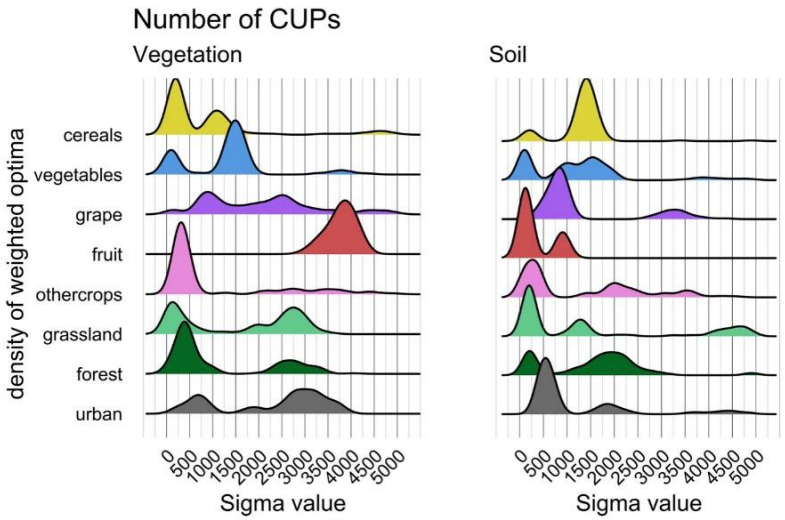


Fig. 6 | Density plot of the weighed optima for the eight land use categories and number of Current Use Pesticides in vegetation and topsoil. Sigma values were the standard deviation of the normal distribution (in metre) which were used for optimization regarding the distance to the prediction site. An optimum was considered as a sigma when the highest multiple R^2 of a model with several sigma compared was reached. Each optimum was additionally weighted with its absolute estimate of the generalized linear model, containing all eight land use categories with a certain sigma combination which resulted in a peak R^2.

concentration and varied the kernel width σ_L for each land type and optimized R^2 for the prediction.

Unfortunately, this results in a nonlinear optimization problem in an 8-dimensional space, which has no simple solution. We therefore used gradient descent methods to find the locally optimal combination of σ_L. We started the optimization algorithm 5000 times with different starting points, e.g. resulting in a total of 176 different local optima for the number of CUPs in vegetation, but we most likely have not found all local optima. For better visual separation we weighted the local optima with their absolute estimate of the resulting model. However, plotting the resulting σ_L reveals that most optima have similar σ_L depending on the land use type (Fig. 6). We caution that we have fit many thousands of linear and generalized linear models during the optimization algorithm. It is therefore not meaningful to compute the p-value for the resulting optimal fits, we therefore only report the corresponding R^2 value.

Overall, land use categories affect the surrounding land much further away than tested basic drift values[30], often for multiple hundred metres (Fig. 6). However, some land use categories (e.g. urban or grassland) showed multiple possible σ, indicating that a certain land use type may have different effects on the surrounding land depending on the distance (See SI for all σ histograms). Land use type urban seems to fit a σ at 750 m and 3000 m for the number of CUPs in vegetation. This could be due to the positive relationship of the occurrence of various agricultural practises near smaller villages (σ 750 m), but when there is a lot of urban land in a larger area (σ 3000 m), agricultural land tends to be absent (larger cities). The categories of crop types must be considered differently, which may be due to the specific CUPs used and their application technique. For vegetation and fruit, a maximum σ of 3750 m was determined. Thus, if CUP contamination is predicted for a non-target site for vegetation during spraying season, 68% of information for fruits contribution is between 0 and 3750 m (see Supplementary information for further details).

Prediction

Regularly spaced prediction points with a distance of 1000 m were placed in the study area. Prediction points in mountain regions that were further from the mountain foot than the measured sample sites were removed (>16.5 km).

The measured number of CUPs and their sum concentrations as independent variables and the optimized combinations of the land use categories and their best explaining σ as dependent variables were used inside a generalized linear model (number of CUPs) or linear model (log of (sum concentration + pseudo constant of 0.01)) for prediction. The predicted values were rasterized to generate a map.

Nature conservation areas

Nature conservation areas (NCAs) shape files were provided by the Bundesamt für Naturschutz[85] and considered if they were located inside the study area. Regularly spaced prediction points with a distance of 200 m were generated inside the NCAs. The same generated glm/lms which were used for the large prediction maps were used to predict the number of CUPs in NCAs. To determine the overall mean number of CUPs inside a NCA, all points inside a NCA were averaged. To generate a map of the "Kleine Kalmit", regular spaced prediction points with a distance of 100 m were generated. Previously mentioned glms were used to predict the number of CUPs and rasterized.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data that were calculated in this study are available in the Supplementary Information. Raw Data and calculated tables also available under https://doi.org/10.5281/zenodo.14832057.

Code availability

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. R Code for generating prediction maps is available under https://doi.org/10.5281/zenodo.14832057. Should any code or raw data files be needed in another format they are available from the corresponding author upon reasonable request.

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K.M.M. and C.H. performed the analytical analysis, validated and prepared the data. K.M.M., J.W. and J.W.S performed the statistical analysis and the landscape regression analysis. All authors discussed the results. K.M.M. wrote the original draft. All authors commented and improved the manuscript and agreed on the final version. C.A.B. supervised the entire project.

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Appendix VI: Paper 6

Effects of annual cropland and season on pollen diversity and pesticide exposure in honey bee colonies

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ABSTRACT

Honey bees face multiple stressors, with cropland expansion, pesticide use, and lack of floral resources being major contributors. However, few studies have jointly investigated altered floral resources and pesticide contaminations in landscapes with increasing dominance of annual crops. Therefore, we studied pollen diversity and current-use pesticide (CUP) residues in pollen samples from 36 honey bee colonies in Lower Franconia, Southern Germany. The nine study sites covered a gradient of annual cropland ranging from 43 % to 97 % within a two-kilometre radius. Pollen was sampled from July to mid-August 2021. Using DNA metabarcoding, a total of 140 different pollen species were identified in 540 pollen samples. Contrary to expectations, pollen richness and Shannon diversity did not decrease with increasing annual cropland area. Instead, richness and diversity showed high temporal turnover and site-specific variation, likely influenced by crop diversity, agro-environmental schemes, and the heterogeneity of surrounding habitats. We conclude that site-specific factors drive the richness and diversity of pollen foraged by honey bees. Among the 16 CUPs detected, fungicides were the most prominent, with notably high concentrations in pollen (3300.5 µg/kg fludioxonil and 971.6 µg/kg azoxystrobin). The probability of detecting fungicides was positively associated with the relative abundance of sunflower pollen, although the detected CUPs were not authorized during flowering. Overall, we observed low pesticide contaminations (39 % of samples), which could be attributed to reduced pesticide applications to the end of the cropping season. Our data indicates that brood in late summer, when long-living winter bees are developing, is less exposed to pesticide residues in pollen compared to earlier phases in the cropping season.

1. Introduction

Insects are declining due to major threats such as climate change, habitat loss, and pesticides (Brown and Paxton, 2009; Hallmann et al., 2017; Müller et al., 2024; Nicholson et al., 2024). Over the last decades, croplands have steadily expanded, and the use of pesticides has continuously increased worldwide (Food and Agriculture Organization of the United Nations, 2025a, 2025b). The Western honey bee (*Apis mellifera* L.) is a generalist using many different plants as nectar and pollen resources (Layek et al., 2020; Lowe et al., 2023). It is an economically important pollinator for a wide range of crops (Khalifa et al., 2021) and forages within a large flight radius of approximately two kilometres (Danner et al., 2016), exposing them to the risk of pesticide contaminations in agricultural landscapes.

Many different pesticides are frequently detected in honey bee colonies, e.g. in honey (El-Nahhal, 2020) and wax (Chauzat and Faucon, 2007). Moreover, the number of pesticides detected in pollen and bee bread has risen strongly over the last thirty years (Rondeau and Raine, 2022). Honey bees adjust their pollen foraging by complex regulatory mechanisms to the need of their colony (Dreller and Tarpy, 2000). The number of larval and adult bees plays a mediating role in this process (Ghosh et al., 2020), as pollen is primarily consumed by younger bees and it is incorporated into larval food (Haydak, 1970). In addition to direct lethal effects of pesticides on honey bee larvae (Lu et al., 2023;

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Manzer et al., 2024; Yang et al., 2020) and adults (Iwasa et al., 2004; Stanley et al., 2015), a multitude of sublethal effects have been reported, such as altered foraging behaviour (Hesselbach et al., 2020; Schuhmann and Scheiner, 2025), impaired long-term memory (El Hassani et al., 2008), and alterations in the gut microbiota (Wu et al., 2022).

Not only pesticides in pollen but also limited pollen quantity and quality can be major stressors for honey bees (Castle et al., 2023). Pollen is the main source of protein, amino acids, fatty acids, as well as micronutrients (Ghosh and Jung, 2017; Haydak, 1970). In general, pollen is known to prolong the lifespan of honey bees (Brodschneider and Crailsheim, 2010). Lack of pollen can cause negative effects, such as the reduced frequency and duration of feeding younger brood. It can even lead to their cannibalization (Schmickl and Crailsheim, 2002, 2001). Furthermore, malnourished honey bees showed delayed development (Brodschneider and Crailsheim, 2010) and depletion of body reserves (Castaños et al., 2023). Physiological changes such as smaller hypopharyngeal glands (Degrandi-Hoffman et al., 2010) and reduced fat body content (Alaux et al., 2010) have also been reported. The fat body, among other important mechanisms, provides insects with immune functions (Arrese and Soulages, 2010). This may play a role in the finding that a lack of pollen led to reduced survival when honey bees were challenged with pathogens (Degrandi-Hoffman et al., 2010; Rinderer et al., 1974; Rinderer and Dell Elliott, 1977). Pollen shortage was further associated with an increased sensitivity to pesticides (Archer et al., 2014; Schmehl et al., 2014).

The nutritional quality of pollen is also important for honey bees. It is generally associated with a high protein content, which can vary largely between 2.9 % and 61 % (Day et al., 1990; Roulston et al., 2000). However, other components can also vary considerably (Odoux et al., 2012). Some authors state the need to consider other nutritional characteristics of pollen such as macronutrients as well (Di Pasquale et al., 2013; Ghosh et al., 2020). Indeed, studies have demonstrated that polyfloral pollen can have positive effects compared to monofloral pollen with a similar protein content. For example, it can result in a higher fat body content, enhanced immune functions in honey bees (Alaux et al., 2010), and increased colony hibernation success (Mainardi et al., 2025). A well balanced polyfloral pollen diet can secure the requirements of a honey bee colony and has the potential to mitigate negative effects by diluting toxic secondary plant metabolites (Bryś et al., 2021; Eckhardt et al., 2014). All of these aspects highlight the need for high quality and diverse pollen resources (Parreño et al., 2022).

During periods of mass flowering oilseed rape, a lower pollen richness has been detected in pollen foraged by honey bees, and colonies located in highly cultivated areas showed nutrient deficiency symptoms (Danner et al., 2017; Dolezal et al., 2019). Additionally, multiple pesticides have been detected in pollen collected by honey bees in agricultural areas (Friedle et al., 2021; Schaad et al., 2023; Zioga et al., 2020). This suggests that honey bees may be challenged by a lack of diverse pollen resources and more pesticide residues in agricultural landscapes dominated by annual crops. Stress caused by low nutrient quality and pesticide residues in combination may have a considerably higher impact on honey bees, particularly in flower-scarce late summer periods. In addition, the rearing of winter bees starts at this time and healthy individuals are of paramount importance for winter survival (Mainardi et al., 2025). Understanding environmental stressors is essential for developing effective strategies to promote honey bee health. However, high-resolution data on combined environmental stressors for honey bees are scarce, especially across agricultural landscape gradients (Willcox et al., 2023).

In this study, we placed honey bee colonies along a gradient of annual cropland, analysed pollen origin in collected pollen samples and assessed residues of 72 current-use pesticides (CUPs), in a period crucial for colony survival between July and mid-August. We focused on high-resolution sampling every third day, to cover the complete pollen foraging pattern and CUP residues. We expected that landscapes with larger proportions of annual cropland would i) provide a reduced overall pollen diversity to honey bees, ii) show a lower pollen species turnover, and iii) lead to an increase of CUP residues in pollen.

2. Material and methods

2.1. Study sites and honey bee colonies

Our study was conducted within a 50 km radius around Würzburg (49°47'11.5"N, 9°57'02.8"E) in Lower Franconia, Germany (Fig. 1A). Nine sites were selected, located along a landscape gradient, ranging from 43 % to 97 % cover of annual cropland within a two-kilometre radius around the centre (Fig. 1B and C). The two-kilometre radius was chosen to cover the typical foraging range of honey bee colonies in Central Europe (Steffan-Dewenter and Kuhn, 2003; Danner et al., 2016). The landscape composition was determined using InVeKoS (Integrated Management and Control System) data from 2021 supplied by the Bavarian State Research Centre for Agriculture (LfL, 2021). Viticulture was not present at the selected sites. Site selection was performed in QGIS (version: 3.32.0-Lima, QGIS Development Team 2024) using the base map from OpenStreetMap Foundation (CC-BY-SA, OpenStreetMap contributors 2024) within the plugin QuickMapServices (version: 0.19.11.1, NextGis 2024). Four honey bee colonies of *Apis mellifera carnica* were established at the centre of each chosen landscape in mid-June 2021 within three consecutive days (Fig. 1D). Colonies were started with artificial swarms (two kg of bee mass) and sister queens (Tobias Nett, Unterschwaningen, Germany) in wooden ten-frame hive boxes (Zander measure) on wax foundations (organic and disease-free Taunus foundations, Bienenzuchtbedarf SEIP, Butzbach-Ebersgöns, Germany). Each colony received seven litres of sugar syrup (Apiinvert, Südzucker AG, Mannheim, Germany) using on-top-feeders and was left to establish for the following three weeks. We replaced three colonies at the beginning of the pollen collection period, one each at sites A, B, and F, because of queen failure, with back-up colonies. These were established in the same way in the departmental apiary of the Julius-Maximilians-Universität Würzburg, Germany. The colonies received one kg of sugar paste (Apifonda, Südzucker AG, Mannheim, Germany) in mid-July.

2.2. Pollen sampling

A total of 540 pollen samples was collected during the study (15 samples per colony x 4 colonies per site x 9 sites) with pollen traps built by the workshop of the university (Figure S1A). Returning pollen foragers brushed off their pollen baskets at the pollen trap, which fell through a grid in a drawer (Figure S1B). The pollen traps were constantly active for the sampling period of six weeks from July to mid-August. Each colony was sampled every third day (Fig. 1D). We stored the amount of pollen samples fitting in a 10 ml flask at −20°C. The remaining pollen was reintroduced as pollen paste to the top of the frames of the originating colony to prevent pollen depletion.

2.3. Pollen metabarcoding

A subsample of each pollen sample (n = 540, Fig. 1E) was individually homogenised and dried for 24 h at 40°C in a drying cabinet (DRY-Line, Avantor VWR, Leuven, Belgium). Three grams of the dry pollen were weighed into 15 ml tubes. To further homogenise the pollen, 10 g of water (autoclaved ultra-pure water, Purelab, ELGA LabWater, Celle, Germany) and a small ceramic ball (Fisher Scientific, Schwerte, Germany) were added and vortexed for 10 min. DNA was extracted using the NucleoSpin Food Kit (Macherey-Nagel, Düren, Germany) (Keller et al., 2015). Next, DNA was amplified following Sickel et al. (2015): PCR for ITS2 markers was performed in three separate 10 µL reactions to avoid PCR bias. For each reaction we employed 1 µL DNA template, 3.34 µL PCR grade water, 5 µL 2x Phusion Master Mix (New England, Biolabs, Ipswich, MA, USA) and 0.33 µM forward and 0.33 µM reverse

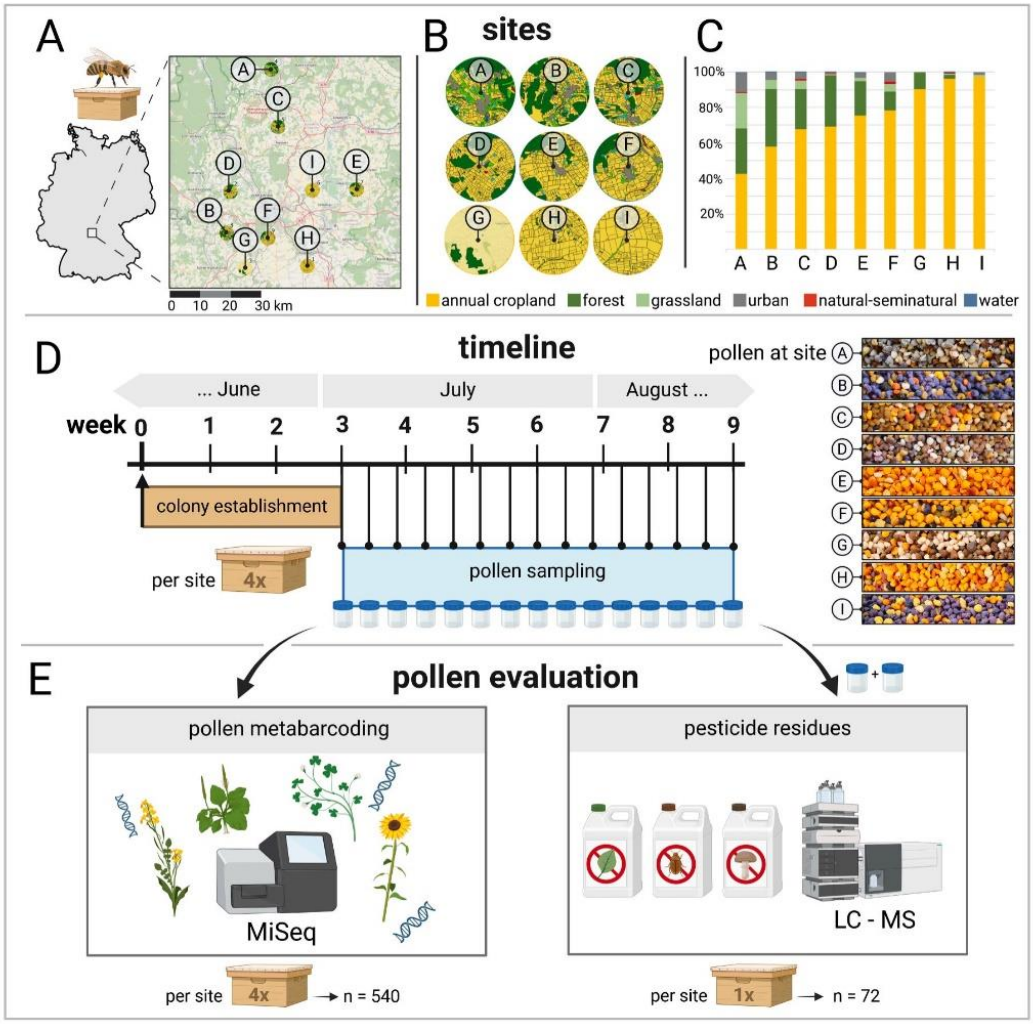


Fig. 1. Experimental set-up with A) the study region around Würzburg, Germany with colony sites, B) the landscape profiles two km around the location of the colonies, and C) the land use gradient of sites (annual cropland in yellow). D) Experimental timeline from mid-June to mid-August with colony establishment and pollen sampling. Exemplary pictures of the collected pollen colours at sites (A-I) are displayed on the right. E) Visualisation of pollen processing for metabarcoding and pesticide residue analysis. The figure was created in BioRender. Manzer, S. (2025) https://BioRender.com/9drsmt2 with elements from QGIS (base map © OpenStreetMap contributors (2024), CC-BY-SA and landscape profiles of the InVeKoS data from the Bavarian State Research Centre for Agriculture (LfL, 2021)).

primers. The forward and reverse primer combination was unique for each sample for specific labelling. The PCR conditions were set to 95°C (2 min) during initial denaturation and to 95°C (20 s) for the 32 cycles of denaturation. For annealing, the conditions were set to 49°C (20 s), for elongation to 72°C (40 s). The final extension step had a setting of 72°C (3 min). We performed normalisation between samples using the SequalPrep Normalisation Plate Kit (Invitrogen, Thermo Fisher Scientific, Frederick, MD, USA) and pooled normalised indexed samples. For quality control, pooled samples were inspected using a Bioanalyzer High Sensitivity DNA Chip (Agilent Technologies, Santa Clara, CA, USA). The samples were quantified with the dsDNA High Sensitivity Assay (Life Technologies GmbH, Darmstadt, Germany) and diluted to 8 ppm. Following, the samples were denatured and spiked with 4.2 % Phix Control Kit v3 (Illumina Inc., San Diego, CA, USA). Sequencing was conducted on the Illumina MiSeq using 2 × 250 cycles v2 chemistry (Illumina Inc., San Diego, CA, USA).

Bioinformatics followed the pipeline available at https://github.com/chiras/metabarcoding_pipeline (version 8c8536b, Leonhardt et al. 2022). The pipeline uses VSEARCH (Rognes et al., 2016) for quality filtering, merging, dereplication and definition of amplicon sequence variants (ASVs) through denoising. Taxonomy was assigned with an iterative approach, with first direct global alignments and a threshold of 97 % against a localized database. This database was created using BCdatabaser (Keller et al., 2020) and a plant species list of Germany. ASV that still remained unclassified were classified against a global vascular plant database (Quaresma et al., 2024; Ratnasingham and Hebert, 2007) as well using VSEARCH global alignments and a threshold of 97 %. Remaining unclassified reads were hierarchically classified using SINTAX (Edgar, 2016) against the global plant database to get taxonomic levels as deep as possible with a threshold of 0.8. ASVs were aggregated at species level, transformed to relative read abundances, and low abundance taxa below 1 % sample contribution were removed from such samples according to positive control sample results. Taxa irrelevant to our study, such as algae and fungi, were filtered out of the dataset. Five of 540 samples were removed due to low throughput (< 500 reads).

2.4. Current-use pesticide analysis

Pollen samples for CUP residue analysis originated from one colony at each site. Two consecutive samples (one gram each) were pooled before the analysis, resulting in an evaluation of the pollen sampled over six days. A total of 72 pollen samples were evaluated (8 pooled samples from one colony at 9 sites, Fig. 1E). Samples were dried in a vacuum freeze dryer at −55°C for 24 h (Alpha 1–4 LSCbasic, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Pollen samples were extracted based on a liquid-liquid extraction method (Honert et al., 2025a) screened for 72 CUPs by liquid chromatography mass spectrometry (HPLC, Agilent LC 1260 Infinity II, Santa Clara CA, USA), followed by electrospray ionization (ESI) and tandem triple quadrupole mass spectrometry (MS/MS, Agilent 6495 C, Santa Clara CA, USA). An internal standard solution (d4-imidacloprid) was used. Detailed methods can be found in the method paper (Honert et al., 2025a).

2.5. Data evaluation and statistics

We analysed the data in R (version 4.3.1, R Core Team 2023) with RStudio (version 2023.6.0.421, Posit team 2023). General data processing was performed using the package suite 'tidyverse' (version 2.0.0, Wickham et al. 2019). We used the package 'ggplot2' (version 3.5.1, Wickham 2016) to create figures. Metabarcoding data was imported and processed using the package 'phyloseq' (version 1.46.0, McMurdie and Holmes 2013).

2.5.1. Alpha and gamma diversity of pollen resources

We evaluated the pollen diversity on a temporal scale, with alpha diversity describing the pollen diversity of individual samples at the sites, and gamma diversity describing the total pollen diversity over time at the specific sites. Diversity was characterised by two measures: species richness and the Shannon diversity index. Species richness was calculated as the number of species per sample (alpha diversity) or per colony over time (gamma diversity). The Shannon diversity index was calculated based on the relative abundance of species per sample (alpha diversity) or per colony over time (gamma diversity) using the package 'vegan' (version 2.6.6.1, Oksanen 2024).

Alpha and gamma diversity were analysed with generalised linear mixed models (glmms) using the package 'glmmTMB' (version 1.1.8, Brooks et al. 2017). The effect of the explanatory variable 'site' was analysed with glmms with random effects for 'sample' (in the case of alpha diversity) as well as 'colony ID' to account for repeated sampling. In the glmms used to analyse the effect of the continuous gradient 'percent annual cropland', random effects for 'sample' (in the case of alpha diversity) and 'colony ID' nested in 'site' were added to incorporate the nested study design. Families were selected according to the data, 'poisson' for species richness (count data) and 'gaussian' for Shannon index (continuous data). Overall significant effects of explanatory variables were explored using the package 'car' (version 3.1.2, Fox and Weisberg 2019) and marginalised model predictions (e.g. marginalised means for different sites or the trend estimations for the annual cropland gradient) using the package 'emmeans' (version 1.10.2, Lenth 2024).

2.5.2. Pollen genera composition

We analysed differences in the composition of pollen genera using a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations and the Bray-Curtis dissimilarity matrix using the package 'vegan' (version 2.6.6.1, Oksanen 2024). In analysing the differences between sites, it was possible to treat each colony as an independent sample of its site. Hence, we aggregated the genus-level relative abundances at colony level prior to the analysis of the sites. To draw conclusions about the gradient of the annual cropland, we aggregated the genus-level relative abundances at site level prior to analysis, since colonies are clustered within site and are not independent samples of the annual cropland gradient. Additionally, we visualised the dominant pollen sources across time and sites for the five dominant pollen sources as the mean relative abundance of the four colonies per sampling timepoint.

2.5.3. Current-use pesticide residues

Data processing of detected CUP residues was done using the software MassHunter Workstation (version 10.0., Build 10.0.707.0, Agilent Technologies 2025). We considered CUP residues to be present if specific ion transitions (MRM, Multiple Reaction Monitoring) were detected and if the response, retention time, and quantifier/qualifier ratio were comparable to those of the standard sample at the same concentrations. To quantify CUP concentrations, an external matrix-matched calibration was applied. For the total number of detected CUPs, residues > limit of detection (LOD) and < limit of quantification (LOQ) were also included, whereas for the CUP concentrations only residues > LOQ were considered. The data was visualised with R studio using the R package 'ggplot2 (version 3.5.1, Wickham 2016).

2.5.4. Relationship between detected current-use pesticide residues and pollen species

We analysed whether the CUP residues in the pollen were related to a certain pollen species using the package 'glmmTMB' (version 1.1.8, Brooks et al. 2017). Because CUP analysis was performed on pooled samples, the relative abundance of pollen species was averaged over the same sample combination. Due to the high number of zero observations in the CUP residue data, a hurdle model was applied to separately analyse the processes underlying the presence/absence of residues and their concentrations when detected. The presence or absence of pesticide residues (zero vs. non-zero) was modelled using logistic regression. The concentrations of pesticide residues (non-zero values) were modelled using a truncated negative binomial distribution to account for the overdispersion of positive values.

3. Results

3.1. Alpha diversity of pollen samples

The pollen richness per sample differed between sites (glmm, $p < 0.001$, Fig. 2A). Based on model predictions, honey bee colonies collected pollen from an average of 9.9 different plant species within a three-day interval (95 % confidence interval (CI) = 9.6 – 10.2) ranging from 6.3 to 13.5 species per site (Table S1). The richness, relative abundance, and species composition within the pollen samples differed, as indicated by the Shannon diversity index (glmm, $p < 0.001$, Fig. 2B). Based on model predictions, the overall mean of the Shannon index per sample was 1.7 (95 % CI = 1.69 – 1.8) with a range of 1.3–2.1 per site (Table S1). We did not find evidence that pollen species richness or Shannon diversity was influenced by the annual cropland gradient (glmm, $p > 0.05$, Fig. 2A and B, Table S2).

3.2. Gamma diversity of pollen

In order to investigate the potential species turnover, we analysed the total pollen richness (Fig. 2C) and Shannon diversity (Fig. 2D) per colony for the full pollen profile over time. A total of 140 pollen species were detected across all samples. Honey bees collected different numbers of pollen species at different sites (glmm, $p < 0.001$). The overall average estimated by the model was 30.5 species (95 % CI = 28.7–32.4), with a range of 19.5–40.8 species per site (Table S3). The Shannon index differed between sites as well (glmm, $p < 0.001$). The overall mean for the Shannon index was 1.7 with a range of 1.3–2.1 per site (Table S3). We found no evidence that total pollen species richness or Shannon diversity per site were influenced by the annual cropland gradient (glmm, $p > 0.05$, Fig. 2C and D, Table S2).

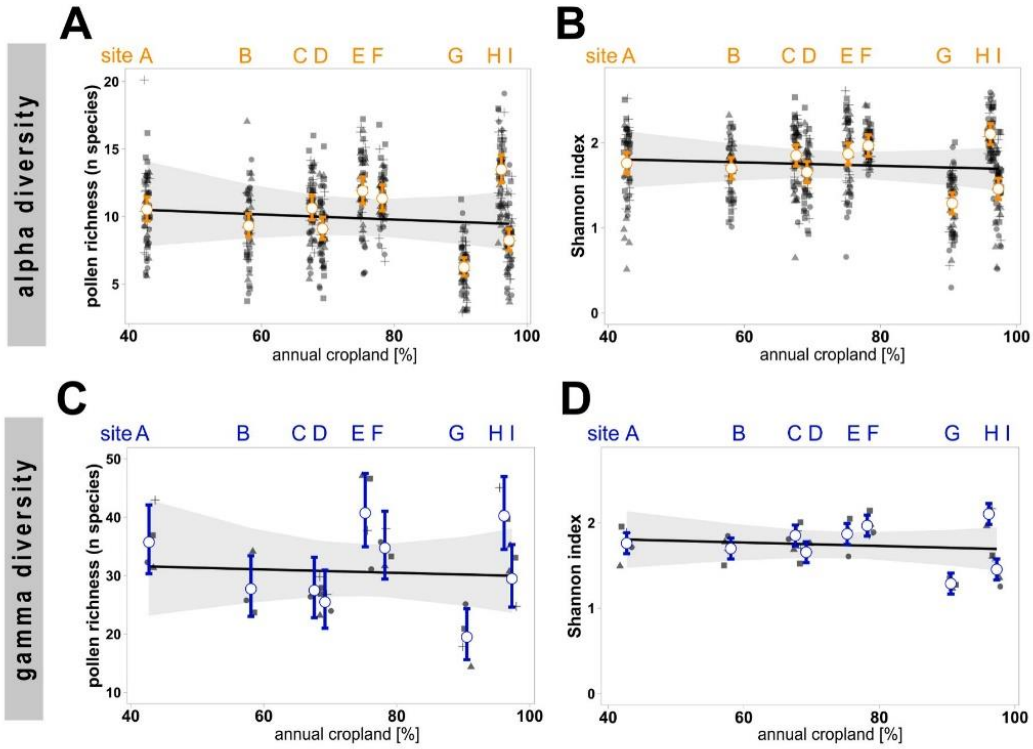


Fig. 2. Effect of annual cropland area on pollen diversity: A) pollen richness and B) Shannon diversity index per sample, and (C) total pollen richness and D) Shannon index per site during the observation time. Different symbols indicate different colonies (n = 4 per site). Horizontal lines show model predictions of the continuous explanatory variable 'annual cropland [%]' with 95 % confidence intervals. Large points in orange or blue display the model predictions of the fixed factor 'site' as marginal mean with the 95 % confidence interval as whiskers.

3.3. Pollen genera composition

A total of 93 pollen genera with a relative abundance greater than one percent per sample were identified. The pollen genera with the highest relative abundance averaged over all sites were *Trifolium* with 16.9 % (range: 3–35 % per site), followed by *Centaurea* with 14 % (range: 3–45 % per site), and *Helianthus* with 8 % (range: 1–30 % per site, Fig. 3A). The composition of pollen genera differed significantly between sites (PERMANOVA, pseudo-F = 21.97, df = 8, p = 0.001) with 87 % of the variance being explained by site differences. All but two sites had at least one pollen genus with a mean relative abundance greater than 20 % (Table S4).

We further analysed whether pollen genera composition could be explained by the annual cropland gradient. We found no evidence that compositional differences among sites were influenced by the cropland area around the colonies (PERMANOVA, pseudo-F = 0.98, df = 1, p = 0.48).

3.4. Temporal turnover of most frequent pollen species

Our data revealed a noticeable turnover in pollen use by honey bees, even within a period of six weeks, from early July to mid-August (Fig. 3B). For illustrative purposes, the five pollen species with the highest relative abundance averaged over all sites were selected: cornflower (*Centaurea cyanus*, 14 %), sunflower (*Helianthus annuus*, 8 %), red clover (*Trifolium pratense*, 8 %), Persian clover (*Trifolium resupinatum*, 6 %) and common poppy (*Papaver rhoeas*, 6 %). The five most prevalent pollen types collectively represented 42.6 % of the relative abundance. A similar pattern of pollen proportions can be observed across sites when the same pollen species were present. For example, the relative abundance of common poppy increased at the beginning of the sampling period and decreased towards the middle (e.g. site B, D, H and I). The collection of Persian clover seemed to start in the first third of the observation period and increased towards the end (e.g. site B, C and G), whereas the relative abundance of sunflower was highest in the middle of the observation period (e.g. site A, D, H, I). Sunflower pollen was present throughout the complete observation time at site E, however, its relative abundance was higher towards the middle and end.

3.5. Current-use pesticide residues

We analysed a total of 72 pollen samples for 72 CUPs, collected from early July to mid-August at nine different sites along a landscape gradient of annual cropland. The analysis revealed the presence of CUP residues in 39 % of the samples. A maximum of five different CUPs per sample were detected in two consecutive samples at site I (97 % annual cropland) in the second half of July.

In total, 16 different CUPs were identified, comprising 11 fungicides, three herbicides, and two insecticides. The most frequently detected CUPs across sites and time were the fungicides azoxystrobin (n (detections) = 18), propamocarb (n = 7) and pyraclostrobin (n = 6, Fig. 4A). Insecticides and herbicides were detected with a maximum of two findings.

The CUPs that occurred at most sites were azoxystrobin (n (sites) = 5), followed by pyraclostrobin (n = 4). Propamocarb was detected at two sites. For the complete observation period, the highest numbers of CUPs (predominantly fungicides) were detected at site I (97 % annual cropland) with 26 detections, site E (75 %) with 15 detections, and site H (96 %) with 9 detections (Fig. 4B). No CUPs were detected at sites D (69 % annual cropland) and G (91 %). We did not detect any

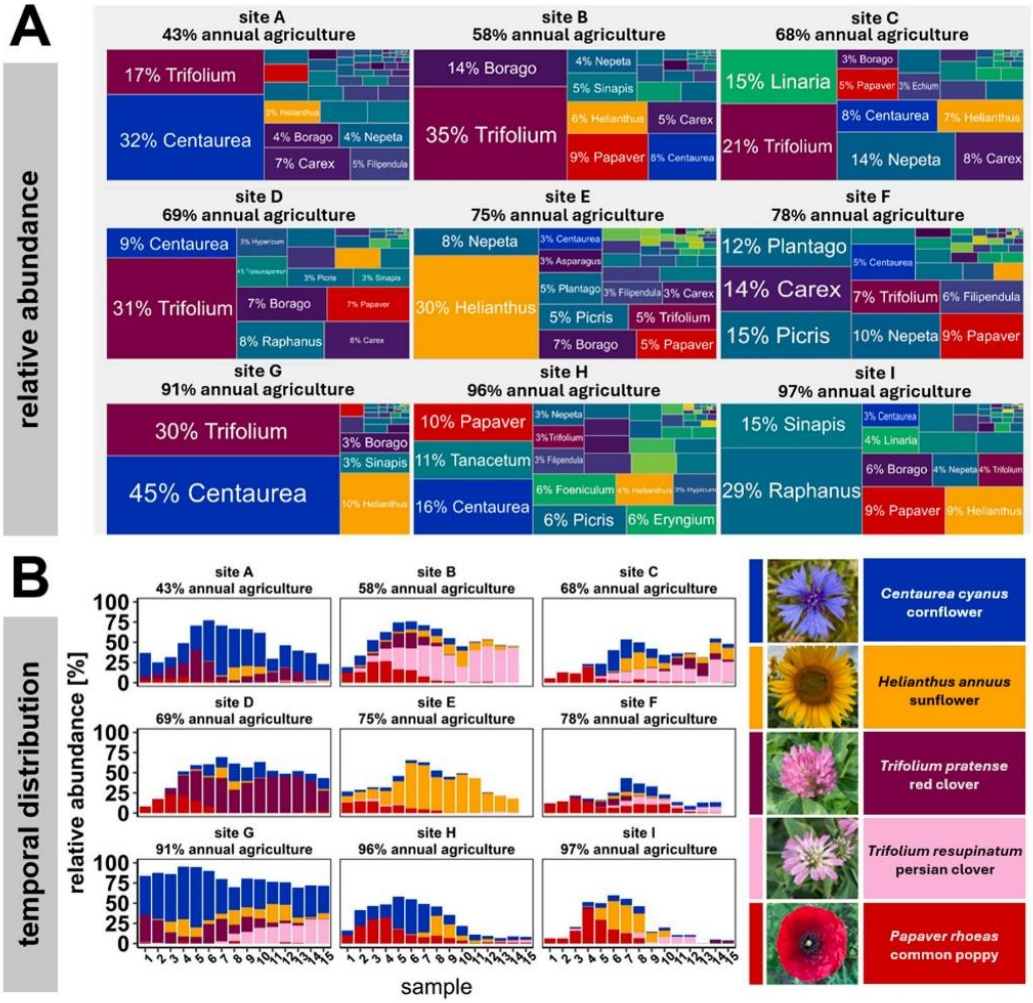


Fig. 3. A) Mean relative abundance of pollen genera at different sites (A-I). Genera with a relative abundance greater than three percent are named in the treeplot, a full list is provided in Table S4. B) Temporal distribution of the five dominant species across all sites, illustrated as the mean relative abundance of four colonies per site and sample. The samples were collected every third day from the beginning of July to mid-August.

insecticides, herbicides and only one to two fungicides at the sites with the smallest annual cropland areas.

The CUPs with the highest residue concentrations were the fungicides fludioxonil and azoxystrobin, with 3300.5 µg/kg pollen and 971.6 µg/kg pollen, respectively (Fig. 4C). Both were detected at site E with the highest concentrations at the end of July with a rapid decrease over a six-day time interval. The corresponding distribution of the relative abundance of the dominant pollen species, averaged over the pooled sample combinations matching the CUP analysis (Figure S2), showed no major visual deviation from the non-pooled relative abundance across all colonies (Fig. 3B).

3.6. Relationship between detected current-use pesticide residues and pollen species

We visually inspected fungicide, herbicide, and insecticide residues per relative abundance of the five dominant pollen species across sites: *Centaurea cyanus*, *Helianthus annuus*, *Papaver rhoeas*, *Trifolium pratense* and *Trifolium resupinatum* (Figure S3). Sunflower (*Helianthus annuus*) was potentially linked to the detected fungicide residues. Higher sunflower pollen proportions were positively associated with the probability of fungicide residue detection (hurdle model, $p < 0.001$). Among samples with fungicide detections, higher proportions of sunflower pollen were also significantly associated with increased fungicide concentrations ($p < 0.001$, Fig. 5).

4. Discussion

Contrary to our expectations, pollen richness and diversity did not decrease with increasing annual cropland area in a landscape. Our data suggests a high pollen turnover during the six-week study period and strong site-specific variation in pollen resources. Overall, we detected mostly fungicides, and CUP residues were unexpectedly low, but peaks occurred in specific landscapes dominated by annual cropland (75 % and 96 % cover in 2 km radius of honey bee colony).

4.1. Pollen diversity differed across sites and showed high pollen species turnover across time

The pollen collected by honey bees differed significantly across sites, containing twice the number of pollen species and about 1.6 times the Shannon index per sample, as well as over the entire observation period.

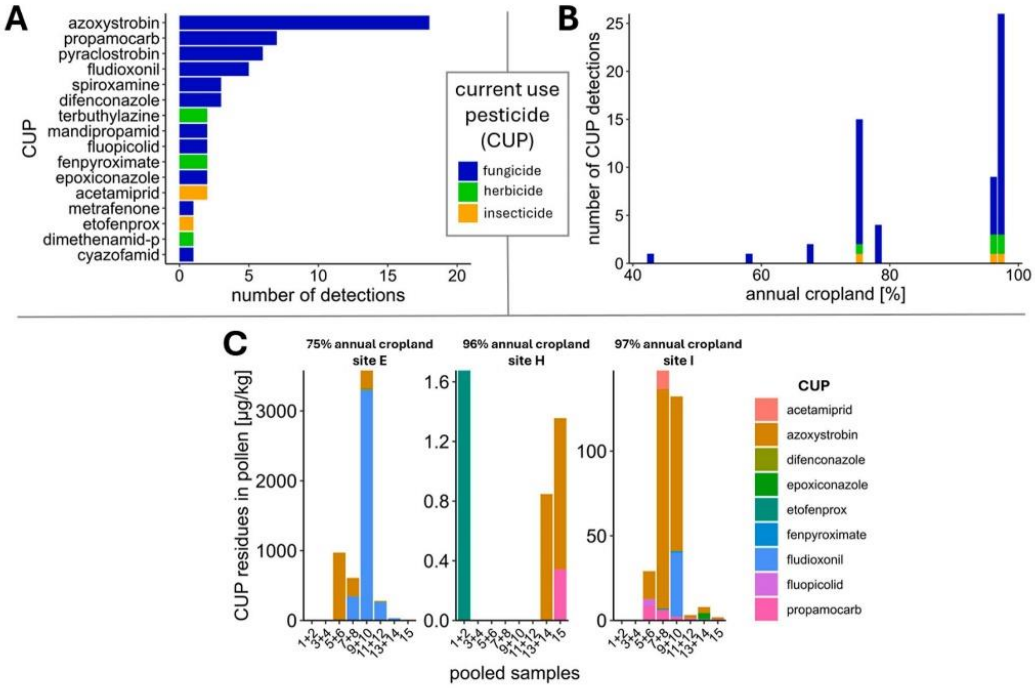


Fig. 4. Detected current-use pesticide (CUP) residues in pollen samples collected during July to mid-August. A) Number of detections per CUP in total and B) number of detections of fungicides, herbicides, and insecticides at different sites. C) Quantified CUP residues at site E, H, and I occurring across the observation period (note the different scales of the y-axis).

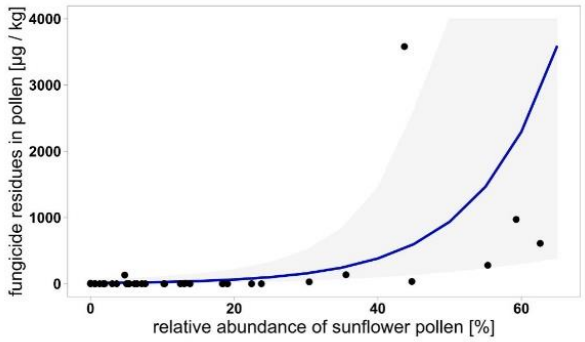


Fig. 5. Fungicide residues in pollen [µg/kg] across the relative abundance of sunflower pollen [%] in the samples. Dots represent detected fungicide residues and model predictions are shown as a blue line with grey confidence interval (hurdle model, shown is the prediction of fungicide residues based on samples containing residues).

Honey bees focused on a small number of pollen species at a time, foraging on an average of 9.9 pollen species within a period of three days. This aligns with the expectation that honey bees prioritise available pollen resources within a landscape (Leponiemi et al., 2023). While studies have found higher species numbers, this was the case for a longer foraging period, for example, Danner et al. (2017) with an average of 23 pollen species per sample for a period of seven days. In studies with a sampling time comparable to our experiment, similar or even lower numbers of pollen richness were reported, e.g. an average of 5.2 pollen species foraged within three days (Simanonok et al., 2021) or a range of five to nine pollen species detected in the majority of samples foraged within three to five days (Avni et al., 2009). Therefore, foraging time is an important factor for the number of detected pollen species.

Across the entire observation period, the total number of identified plant species was considerably higher than in individual samples, averaging 30.5 pollen species across all sites. This suggests that honey bees collected pollen from a wide variety of plant species over time, reflecting a rapid turnover. A notable shift with an increase and/or decrease in relative abundance of pollen species per sample became evident across sites for species such as sunflower (*Helianthus annuus*), common poppy (*Papaver rhoeas*), red clover (*Trifolium pratense*), and Persian clover (*Trifolium resupinatum*). This highlights the highly dynamic nature of the resource landscapes exploited by honey bee colonies. The observed patterns may display the changes in blooming vegetation over time, as we observed similar shifts in on- and offset of pollen species across several sites. Therefore, fine-scale pollen metabarcoding of pollen resources sampled every third day could be a promising tool for future research to depict flowering phenology and related resource exploitation by honey bees.

4.2. No pollen diversity decline along the annual cropland gradient

No evidence was found that the variation in pollen richness or Shannon diversity between sites (per sample and across the full observation period) could be attributed to the annual cropland gradient. Our study focused on the six-week period from July to mid-August. An effect of the landscape gradient may have become apparent when observing longer timeframes, as pollen richness has been shown to vary throughout the year in long-term studies with less frequent sampling (Brodschneider et al., 2019; Malagnini et al., 2022). However, in an earlier study conducted in the same study region as our experiment (Würzburg, Germany), Danner et al. (2017) similarly observed no influence of landscape diversity on pollen richness or Shannon diversity collected by honey bees. The authors suggest a possible compensation strategy of honey bees by prioritising pollen foraging in the remaining semi-natural habitats and expanding their foraging range in less diverse

landscapes. Indeed, Steffan-Dewenter and Kuhn (2003) reported significantly larger pollen foraging distances of honey bees in structurally simple landscapes, compared to complex landscapes, for the beginning of May to the end of July. Interestingly, in our study, the site with the second highest annual cropland (96 %) exhibited the highest pollen richness and Shannon index. In contrast, the lowest diversity was observed at two sites with similar proportions of annual cropland (91 % and 97 %, respectively).

In addition to differences in pollen diversity, the composition of pollen genera also varied across sites, with no evidence that this variation was explained by the proportion of annual cropland. There was also no visible trend in the same dominant pollen genera in sites dominated by annual cropland. In general, the pollen genera *Trifolium*, *Centaurea*, and *Helianthus* had the largest overall relative abundance with *Trifolium* and *Centaurea* being present at all sites. *Trifolium* is a major pollen source for honey bees and other pollinators (Lanuza et al., 2025; Sponsler et al., 2020; Stoner et al., 2022) and has been described as one of the most prominent floral nectar sources (Baude et al., 2016). Different species of *Trifolium* can occur in different types of grasslands (Tserendorj et al., 2021) and in cultivated mixtures with several grass species for livestock fodder (Lüscher et al., 2014). *Centaurea* mainly grows in oilseed rape and wheat fields and, due to its dependence on arable land, has been used as a biological indicator of species richness in arable fields (Bellanger et al., 2012). *Helianthus annuus* is commonly cultivated for oil production and the harvested sunflower area has more than doubled from 2016 to 2021 in Germany (Food and Agriculture Organization of the United Nations, 2024). Furthermore, the three pollen genera *Trifolium*, *Centaurea*, and *Helianthus* are part of seed mixtures that are specifically designed for flower strips or fields subsidised in Germany (Bavarian State Research Centre for Agriculture, 2023). Therefore, cultivated crops with their weeds and flower strips have a major influence on pollen availability for honey bees. The presence of several dominant pollen genera in different agricultural crops indicates that the explanatory variable 'percentage annual cropland' might be too general to draw detailed conclusions on pollen (non-)availability.

In addition, annual cropland practices can have a notable impact. Weeds are important pollen sources (Odoux et al., 2012; Requier et al., 2015, Bertleff et al., 2025 – in press) and the efficacy of weeding methods can vary considerably (Cirujeda et al., 2003), influencing pollen availability. Furthermore, other agricultural practices, such as the recommended early mowing of *Trifolium repens* (white clover) in its vegetative stadium before flowering to achieve a high protein content in the fodder for livestock (Solati et al., 2017), shape the availability of pollen in the landscape. Different crops and agricultural practices are likely to have contributed considerably to the significant variation observed between sites. This highlights the opportunity and necessity to contribute to the well-being of pollinators with insect-friendly annual cropland practices and underlines the importance of research with precise data on resources available for honey bees.

4.3. Possible impact of honey bee foraging strategies on pollen diversity

In addition to the general pollen availability in the landscape, honey bee foraging strategies may have contributed to observed differences in collected pollen diversity. There could be two honey bee strategies to ensure a good pollen nutrition: a) to concentrate on large fractions of beneficial pollen or b) to compensate for unbeneficial pollen by increasing the overall diversity. The nutritional value of different pollen species can vary considerably (Day et al., 1990; Odoux et al., 2012; Roulston et al., 2000). Therefore, it is crucial to consider not only the quantitative differences in pollen species but also their origin and nutritional profiles. For example, site G is considered to have the lowest pollen diversity and could be interpreted as non-beneficial to honey bees. However, two genera with high relative abundance, *Centaurea* (45 %) and *Trifolium* (30 %), are known for their high protein content of 20.6–23.7 % (Tasei and Aupinel, 2008) and 27.1–27.7 % (Salonen et al., 2021), respectively. Furthermore, *Trifolium* is considered to have a well-balanced elemental composition (Filipiak et al., 2017). Site G may have provided an exceptional good nutritional profile. Especially in comparison to site E, which ranks high in pollen diversity measures but also has a high relative abundance of *Helianthus* pollen (30 %), which has a protein content of only 14.4 % protein (Tasei and Aupinel, 2008), and is even considered to negatively affect bee growth due to its scarcity of phosphorus (Filipiak et al., 2017). However, we cannot draw reliable estimates from pollen metabarcoding to weight proportions and nutrient profiles. Additionally, other nutritional aspects such as macronutrients or the protein-lipid ratio should also be considered (Di Pasquale et al., 2013; Ghosh et al., 2020; Vaudo et al., 2024, 2020). Environmental studies that combine pollen metabarcoding, nutritional assessments, and observations of cultivated crops and practices could be highly beneficial in providing knowledge on the impact of annual cropland on honey bee health.

Another foraging strategy that may explain consistently low numbers of pollen species is the ability of honey bee colonies to balance nutrient deficiencies by selectively collecting pollen that complements their existing stores (Hendriksma and Shafir, 2016; Zarchin et al., 2017). Further it may be assumed that stored bee bread provides sufficient diversity, thus reducing the need for energy-costly foraging strategies and, consequently, lowering pollen diversity in samples. The stored bee bread is rapidly consumed, with 70 % within the first three to five days and the remaining stores within two to three weeks (Roessink and van der Steen, 2021). This suggests that the honey bee colonies in our study consumed their entire pollen yield at least two to three times during our six-week observation period. Therefore, we observed at least two or three complete pollen profiles that had been collected. In conclusion, it is unlikely that the constantly lower pollen diversity at some sites has been caused by the previously collected pollen diversity.

It may be possible that a rather low pollen diversity is also supported by the foraging strategies of honey bees. Honey bees focus on one pollen source per foraging flight (Leonhardt and Blüthgen, 2012). Using dance communication for floral patches, returning foragers advertise profitable pollen patches in a higher intensity compared to less profitable ones. Their dance instructions are consequently observed by more new foragers, which leads to a focus on the most profitable and most-communicated patches, potentially resulting in fewer foraged pollen species. However, while this pattern was observed in landscapes with few mass-flowering crops, Nürnberger et al. (2019) further demonstrated that in intensively managed landscapes dominated by mass-flowering crops with a low floral resource diversity, dance communication enabled colonies to collect a greater pollen diversity of species which may have been present at low abundances. In conclusion, waggle dance enables specific recruitment, which helps colonies to compensate for low-diversity landscapes and thus maintain pollen diversity. However, different landscapes may trigger different honey bee reactions.

It is important to note that foraging strategies can differ among bee species and therefore also the effect of landscape composition. While, for example, bumble bees visit multiple plant species during a single foraging flight (Leonhardt and Blüthgen, 2012) other wild bees are flower-constant, similar to the honey bee (Layek et al., 2025). Research conducted on honey bees cannot be directly applied to wild bee species, highlighting the urgent need for more targeted studies in this area.

4.4. High fungicide residues in pollen

Our data showed an upward trend in CUP detections with the percentage of annual cropland. Further, we did not detect any insecticides, herbicides, and only one to two fungicides at the sites with the smallest annual cropland areas, which supports the assumption of a lower CUP load with less annual cropland land use. Overall, we detected fungicides most frequently, which is consistent with the results of other studies analysing honey bee-collected pollen (e.g. Cappellari et al. 2024, Tosi

et al. 2018, Friedle et al. 2021). In general, cumulative detections of different fungicides in honey bee colonies have increased sharply over the last years, especially in foraged pollen (Rondeau and Raine, 2022). Fungicide applications are carried out protectively before a disease spreads and are related to high humidity and warm temperatures. A rainy season can be a reason for fungicide applications. In Würzburg, which was the approximate centre of the research area, a total of 18 days with rain (13 days with a precipitation > 1 l/m^2) were recorded in July, and 20 days (10 days $>$ l/m^2) in August 2021 (Meteostat, 2025).

We identified two fungicides at maximum concentrations of 3300.5 µg fludioxonil/kg pollen and 971.6 µg azoxystrobin/kg pollen, likely because of recent spraying. For fludioxonil, the lethal dose (LD_{50}) – the dose at which 50 % of exposed honey bees are expected to die – has been described as > 25 µg/bee (National Center for Biotechnology Information, 2025b). Calculating the pollen hazard quotient (PHQ, measure for pollen toxicity of contaminated pollen for honey bees) with the formula 'µg residue per kg pollen / LD_{50} of the respective residue' (Stoner and Eitzer, 2013), detected residues of fludioxonil resulted in a PHQ of 132 and would be categorised as moderate (PHQ > 50–500). For azoxystrobin, LD_{50} values are described to be > 200 µg/bee (National Center for Biotechnology Information, 2025a), resulting in a PHQ of 4.9. This PHQ would be categorised as low (PHQ < 50). However, this categorisation refers to direct mortality and does not consider negative sublethal effects or synergistic effects which may occur because of pesticide mixtures or accumulating effects because of residue intake over time.

Our data showed a residue detection dynamic of high peaks, with maximum residues several times higher than those observed in the previous and following six days. This is in agreement with Friedle et al. (2021), where daily pollen samples also showed peaks, which were several times higher than the previous and following samples. This highlights the importance of small sampling intervals to show clear peaks of pesticide residues. Due to the rapid consumption of beebread, the exposure of larvae and nurse bees with nondegraded pesticide residues may be a justified worst-case assumption (Roessink and van der Steen, 2021). Studies investigating the pesticide exposure of honey bees should be carried out over a long period of time with frequent sampling occurring at least on a weekly basis, as we detected well-pronounced CUP peaks within 6-day samples.

4.5. Fungicides are positively associated with sunflower pollen

The presence of fungicide residues, as well as their concentrations, were positively related to the relative abundance of sunflower pollen in the pollen samples. The fungicide fludioxonil occurred at the site with the highest overall relative abundance of sunflower pollen (30 %), however fludioxonil is only approved as seed treatment in sunflowers in Germany (BVL, 2024b).

The CUP most frequently detected was the fungicide azoxystrobin. It is part of 79 authorised fungicide formulations in Germany for various arable, vegetable, and ornamental crops, but not for sunflowers (BVL, 2024a). Similar to our study, Schaad et al. (2023) detected azoxystrobin during the flowering period of sunflowers in Switzerland, without authorisation for this use. Honert et al. (2025b) report a notable decrease in multiple CUPs from field margins in vegetation and topsoil, therefore our detected residues may be explained by drift from other treated crops to sunflowers, honey bee attractive flowering weeds, or flowering strips along field margins. Further analysis with sorted pollen species before the analysis of CUPs can support our findings and provide valuable insight into the origin of pollen contaminations and possible drift.

4.6. Current-use pesticide residues depend on the sampling period, cultivated crop stages at the given time, and methods of analysis

We detected surprisingly low numbers of CUPs, with a total of 16 different CUPs identified and a maximum of five CUP residues per sample in the second half of July at site I (97 % annual cropland). In general, a high variation of detected pesticides in pollen or bee bread has been reported, e.g. 19 (Chauzat et al., 2006), 29 (Friedle et al., 2021), 30 (Schaad et al., 2023), 73 (Böhme et al., 2018) or even 75 different pesticides (German Bee Monitoring Programme, 2021). When comparing the numbers of pesticides, it is important to consider the method applied for analysis. For example, extraction methods and applied quantification limits can vary between studies. Due to analytical methods, our analysis included 72 CUPs, covering one-quarter of approved pesticides in Germany ($n = 281$), which, however, also include biological pesticides or agrochemicals, such as pheromones or plant growth regulators (BVL, 2025). Other studies covered more pesticides and metabolites (e.g. 475 by the German Bee Monitoring Programme 2021 or 262 by Friedle et al. 2021). Therefore, we assume that there may be more CUP residues present in our samples than detected. Furthermore, in contrast to other studies, where, for example, five grams of pollen was used for pesticide analysis (Böhme et al., 2018), we extracted 0.1 g of the pollen sample, which could result in fewer detections.

Sampling methods can also contribute to the different numbers of pesticides detected in the studies. We have specialised on short time intervals with fine-scale pollen sampling every third day within a narrow area of 50 km. Extending the duration of the observation period and expanding the observation area can increase the number of pesticides detected. For example, the 75 different pesticides detected by the German Bee Monitoring Programme (2021) originated from bee bread samples collected throughout Germany in spring, summer, and autumn, or the 73 CUPs detected by Böhme et al. (2018) were observed in a five-year study.

We were surprised to find a low contamination rate, with 39 % of pollen samples containing CUP residues, while other studies detected pesticide residues in 91 – 100 % of samples of beebread (German Bee Monitoring Programme, 2021), pollen foraged by honey bees (Friedle et al., 2021), or pollen sampled from plants (David et al., 2016). In addition, pesticides have been detected in 97 – 100 % of topsoil, vegetation and insect samples (Honert et al., 2025b; Brühl et al., 2021). The most important factors in detecting pesticides in pollen may be the sampling period and the pollen resources available at the observation time. Studies with a higher temporal resolution, also conducted in Germany, report maximum concentrations and most detected pesticides per sample during April, May, as well as the second half of June, while during June or July the lowest pesticide concentrations or detections were reported (Böhme et al., 2018; Friedle et al., 2021). Our pollen was sampled from July to mid-August, even extending the previously described season in which low pesticide residues have been detected. Low pesticide detections in pollen at the end of the summer are also in line with a study performed over two years in the flowering period (April to September) in Denmark, also showing a decrease in pesticide detections and pesticide diversity to the end of the season (Murcia-Morales et al., 2021).

The reason for the importance of timing in pesticide assessment is the link between their use at specific stages of cultivated crops or fruit trees. In April, maximum values of two fungicides, fluopyram (4050 µg /kg pollen) and tebuconazole (4530 µg/kg pollen), have been detected in samples with high proportions of *Prunus* sp. (± 77 % stone fruit) and *Pyrus* sp. pollen (± 18 % pome fruit), assuming that this ratio was likely present and collected from local fruit cultivations (Friedle et al., 2021). Neonicotinoids, e.g. thiacloprid (37 µg /kg bee bread) have been observed during the flowering of oilseed rape in May (Schaad et al., 2023) and high pesticide contaminations were detected in the pollen of *Brassicae* sp. and *Vitis vinifera* L. (grapevine) by Böhme et al. (2018). Furthermore, spraying against aphids after apple bloom in May (Favaro et al., 2019), or treating maize seedlings in June (Schaad et al., 2023) can lead to pollen contaminations. All these practices and flowering time points have been outside our observed time frame and underline the

strong seasonal variation in pesticide residues. The pesticide residues along the annual cropland gradient during April to July may have been higher and a correlation could have been more pronounced. Indeed, Cappellari et al. (2024) reported an increase in the PHQ with urban and agricultural areas from April to July, while the PHQ was low and independent of landscape composition at the end of the season from August to September. Our data indicates that even in landscapes with high proportions of annual cropland, the pesticide contaminations of pollen may be lower than recorded in the vegetation in proximity to agricultural crops, where e.g. a local peak in July with approximately 9 different pesticides was detected (Honert et al., 2025b). If this finding is confirmed in other landscapes and years, it would indicate that late-summer colonies are not as much exposed to pesticides in pollen as during spring and early summer. Given the multiple other stressors and factors influencing honey bee colony winter survival, such as Varroa infestation (van Dooremalen et al., 2012) or summer weather conditions (Calovi et al., 2021), a lower pesticide exposure of winter bees via pollen would be beneficial for hibernation success. More research combining detailed data on grown crops, their individual flowering timepoints in a certain region, a close investigation of weeds as pollen and nectar sources and spraying timepoints, coupled with pesticide residue investigations of pollen foraged by honey bees, would further deepen the understanding of the potential hazards of annual cropland on honey bees.

4.7. Context of the annual cropland gradient in this study

As previously discussed, our data and the conclusions drawn from the landscape gradient in annual cropland cannot be generalised throughout the year. Furthermore, it is important to consider the context of the study region and country, as the effects of landscape variables can vary greatly. For example, Larsen and Noack (2017) detected an increase in insecticide use with increasing field sizes in California, but a reduction in pesticide use in larger farms in China has been reported (Gao et al., 2021). There can also be different pesticide regulations with different effects on insects, for example, 72 pesticides used in more than one quarter of all applied pesticides in the US are banned in the EU (Donley, 2019). Therefore, the gradient of annual cropland sampled from July to August may lead to different results in pesticide residues and their toxicity to insects in other countries. Further research is necessary to gather more information on environmental stressors for honey bees on a landscape scale.

5. Conclusion

In contrast to our expectation, increasing annual cropland proportions did not lead to a lower richness or diversity (Shannon index) of foraged pollen, possibly because of energy-costly compensation strategies like increasing foraging distances. Besides the creation and protection of important semi-natural habitats, landscape-level crop composition, the implementation of flower strips, and other management factors can lead to differences in available pollen, highlighting the possibility to help honey bees to sustain a high nutritional quality diet in agri-environmental schemes.

Pollen analysis revealed predominantly fungicide residues, with few detected herbicide and insecticide residues. Higher fungicide residues were positively associated with a higher relative abundance of sunflower pollen, potentially caused by drift. Overall, detected CUP residues were unexpectedly low, which could be explained by the sampling period from July to mid-August after the main pesticide application period. Our results suggest that long-lived winter bees are less exposed to pesticide residues in pollen during their development than short-lived summer bees.

CRediT authorship contribution statement

Ingolf Steffan-Dewenter: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Ricarda Scheiner:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Giulia Mainardi:** Writing – review & editing, Investigation. **Carsten A. Brühl:** Writing – review & editing, Resources. **Carolina Honert:** Writing – review & editing, Validation, Methodology. **Alexander Keller:** Writing – review & editing, Methodology, Formal analysis. **Douglas Sponsler:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Sarah Manzer:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis.

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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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2025.109987.

Data availability

Data will be made available on request.

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Appendix VII: Declaration of generative AI technologies in the writing process

During the revision of the abstract, introduction, methods, discussion, synthesis, and outlook chapters, the author used DeepL, ChatGPT-4, and Grammarly selectively to improve English grammar, spelling, and phrasing. Gemini was used to adapt Figures (Figure 2), initially built by the author in biorender.com. All content generated with these tools was carefully reviewed and edited by the author, who takes full responsibility for the final version of the text.

Appendix VIII: Declaration

I, the undersigned author of this work, hereby declare that I independently wrote this thesis with the title “Exposure of insects to current-use pesticides in agricultural landscapes: temporal occurrence and spatial distribution, and implications for risk assessment”. All used resources and references are specified in this work. Contributions of co-workers and co-authors have been clearly identified.

I did not use the assistance of a doctoral consultant (or a similar person) in return for payment.

I did not, and have never, submitted this PhD thesis in identical or similar form for scientific examination elsewhere.

I am aware that a violation of the above-mentioned points can lead to a withdrawal of the doctoral degree and additional legal consequences

_____ _____

Place, Date Carolina Sophia Maria Honert

Appendix IX: Curriculum Vitae

CAROLINA HONERT

EDUCATION

08/2020 – 05/2026	**Dissertation in natural sciences (PhD),** University of Kaiserslautern-Landau, Landau, Germany
10/2017 - 08/2019	**Master of Science Biology**, Technical University of Munich, Freising, Germany
10/2014 - 07/2017	**Bachelor of Science Biology,** Technical University of Munich, Freising, Germany
08/2005 - 08/2013	**Abitur,** Königin-Luise-School, Cologne, Germany

PROFESSIONAL EXPERIENCE

08/2020 – actual	**Research associate**, University of Kaiserslautern-Landau, Landau, Germany
02/2020 – 06/2020	**Employee**, Agricultural businesses (Würzburg, Germany and Oppikon, Switzerland)
09/2016 – 04/2018	**Student assistant,** Bavarian State Institute for Forest and Forestry Sciences, Freising, Germany
04/2015 – 03/2017	**Student assistant,** Technical University of Munich, Freising, Germany

PEER-REVIEWED PUBLICATIONS

INCLUDED IN THIS THESIS

Bakanov, N.; **Honert, C.**; Eichler, L.; Lehmann, G. U. C.; Schulz, R.; Brühl, C. A. (2023): A new sample preparation approach for the analysis of 98 current-use pesticides in soil and herbaceous vegetation using HPLC-MS/MS in combination with an acetonitrile-based extraction. In Chemosphere 331, p. 138840. DOI: 10.1016/j.chemosphere.2023.138840.

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FURTHER STUDIES

Lupp, G.; Kantelberg, V.; Förster, B.; **Honert, C.**; Naumann, J.; Markmann, T.; Pauleit, S. (2021) Visitor Counting and Monitoring in Forests Using Camera Traps: A Case Study from Bavaria (Southern Germany). In: Land 7, 736. DOI: 10.3390/land10070736

Lupp G., Förster B., Naumann J., **Honert C.,** Kantelberg V., Koch M., Pauleit S. (2016): Using trigger trail cameras for visitor monitoring – Applications in Bavaria. In : Monitoring and Management of Visitors in Recreational and Protected Areas. Abstract Book, 26–30 September 2016, Novi Sad, Serbia. Available online at https://mediatum.ub.tum.de/1368225.

Lupp, G.; Förster, B.; Kantelberg, V.; Markmann, T.; Naumann, J.; **Honert, C.**; Koch, M.; Schreiber, R.; Pauleit, S.(2016) Assessing the Recreation Value of Urban Woodland Using the Ecosystem Service Approach in Two Forests in the Munich Metropolitan Region. In: Sustainability 8, 1156. DOI: 10.3390/su8111156

Lupp, G.; Kantelberg, V.; Förster, B.; Naumann, J.; **Honert, C.**; Schreiber, R.; Pauleit, S. (2016): Vorsicht – Kamera! Besuchermonitoring mit Wildkameras. In: LWF aktuell 111, 14-16.

CONTRIBUTIONS TO SCIENTIFIC CONFERENCES

05/2026	**NBS Dialogue Forum: Insect Conservation in Practice – Lessons Learned from Projects under the Federal Programme for Biological Diversity** (NBS-Dialogforum: Insektenschutz in der Praxis – Erkenntnisse aus den Projekten im Bundesprogramm Biologische Vielfalt), Bonn, Deutschland
04/2026	**Expert Event on Pesticide Inputs and Biodiversity** (Fachveranstaltung Pflanzenschutzmitteleinträge und Biodiversität) Duisburg, Deutschland
11/2025	**West German Entomologists' Conference** (Westdeutsche Entomologen Tagung), Düsseldorf, Germany
05/2025	**SETAC Europe 34th Annual Meeting,** Sevilla, Spain
09/2023	**SETAC GLB e.V. GDCh-FG Environmental Chemistry & Ecotoxicology Joint Annual Conference,** Muttenz, Switzerland
10/2023	**Networking of scientific actors in the field of insect protection** Vilm, Germany
08/2023	**Setac YES Meeting**, Landau, Germany
09/2021	**South-west German Entomology Day** (Südwestdeutscher Tag der Insektenkunde**): "Measures and success in the protection of insects**", Karlsruhe, Germany

CO-SUPERVISED THESESES, RESEARCH PROJECT COURSES, AND INTERNSHIPS

Thesis, M.Sc. Environmental Pollution Management (Ecotoxicology)

Depth- and Time-Dependent Fate and Transfer of Current-Use Pesticides in Different Agricultural Soils

Thesis, M.Sc. Environmental Pollution Management (Ecotoxicology)

Pesticide Exposure of Insects in Flower Strips on Agricultural Field Areas in Landau in der Pfalz and Surrounding Areas, Germany

Thesis, M.Sc. Environmental Pollution Management (Ecotoxicology)

Possible effects of pesticide drift on earthworms and collembolans in different distances to agricultural land

Thesis, M.Sc. Environmental Pollution Management (Ecotoxicology)

Pesticide residue analysis of pollen collected by Osmia bicornis exposed to field pesticide concentrations and effects on development

Thesis, B.Sc. Conservation Biology and Environmental Chemistry

Investigation of pesticide exposure in Osmia bicornis during larval development by pollen supply

Thesis, B.Sc. Conservation Biology and Environmental Chemistry

Long distance transport of CUPs in Germany – Precipitation and seasonal distribution

Thesis, B.Sc. Forensic Science

Pesticide exposure in insects Method validation and taxonomic properties of uptake and degradation of pesticides with distance over time

Research project course (RPC), M. Sc. Environmental Pollution Management (Ecotoxicology)

Develop of pesticide extraction methods based on QuEChERS from Apis mellifera pollen

Research project course (RPC), M. Sc. Environmental Pollution Management (Ecotoxicology)

Oral Toxicity of frequently detected pesticides in vegetation samples in proximity to agricultural fields on Caterpillars

Research project course (RPC), M. Sc. Environmental Pollution Management (Ecotoxicology)

Method development for the extraction of 98 current-use pesticides in Flower Matrix using HPLC-MS/MS

Internship, Research project course (RPC), M. Sc. Environmental Pollution Management (Ecotoxicology)

Pesticide contamination overview of two pollinating insects in agricultural landscape using HPLC-MS/MS analysis

Internship, Research project course (RPC), M. Sc. Environmental Pollution Management (Ecotoxicology)

Pesticides in environmental matrices to assess insect exposure

Internship (Ecotoxicology)

Pesticide residues in insect associated environmental matrices

ETX 5b Effects of Chemical Stressors II, M. Sc. Environmental Pollution Management (Ecotoxicology)

Investigations on Pesticide Residues on Playgrounds

ETX 5b Effects of Chemical Stressors II, M. Sc. Environmental Pollution Management (Ecotoxicology)

Investigations on Pesticide Residues in Pollen

ETX 5b Effects of Chemical Stressors II, M. Sc. Environmental Pollution Management (Ecotoxicology)

Investigations on Pesticide Residues in Flowers

RESEARCH COLLABORATIONS

2023	**University of Aachen**, Aachen, Germany
2020	**University of Würzburg**, Würzburg, Germany

COURSES

2023	**Video editing Course**, Landau, Germany
2023	**Scientific Writing Course,** Landau, Germany
2021	**QQQ-LC/MS Technics and Software Operations**, Waldbronn, Germany
2020	**GIS Course,** Landau, Germany
2018	**Stabile Isotop Course,** Budweis, Czech Republic

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